(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁷: C12N 15/11, A61K 31/713

(21) International Application Number: PCT/US01/23874

(22) International Filing Date: 30 July 2001 (30.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/629,644   31 July 2000 (31.07.2000)   US

(71) Applicant *(for all designated States except US)*: **ISIS PHARMACEUTICALS, INC.** [US/US]; Carlsbad Research Center, 2292 Faraday Avenue, Carlsbad, CA 92008 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **COWSERT, Lex, M.** [US/US]; 3008 Newshire Street, Carlsbad, CA 92008 (US). **WYATT, Jacqueline** [US/US]; 1065 Hymettus Avenue, Encinitas, CA (US). **FREIER, Susan, M.** [US/US]; 2946 Renault Street, San Diego, CA 92112 (US). **MONIA, Brett, P.** [US/US]; 7605 Nueva Castille Way, La Costa, CA 92009 (US). **BUTLER, Madeline, M.** [US/US]; 15951 Avenida Calma, Rancho Santa Fe, CA 92091 (US). **MCKAY, Robert** [US/US]; 277 Caminito Pescado #73, San Diego, CA 92116 (US).

(74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, Box 457, Spring House, PA 19477 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ANTISENSE MODULATION OF PTP1B EXPRESSION

(57) Abstract: Compounds, compositions and methods are provided for modulating the expression of PTP1B. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding PTP1B. Methods of using these compounds for modulation of PTP1B expression and for treatment of diseases associated with expression of PTP1B are provided.

ANTISENSE MODULATION OF PTP1B EXPRESSION

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of PTP1B. In particular, this invention relates to compounds, particularly antisense oligonucleotides, specifically hybridizable with nucleic acids encoding PTP1B. Such oligonucleotides have been shown to modulate the expression of PTP1B.

BACKGROUND OF THE INVENTION

The process of phosphorylation, defined as the attachment of a phosphate moiety to a biological molecule through the action of enzymes called kinases, represents one course by which intracellular signals are propagated resulting finally in a cellular response. Within the cell, proteins can be phosphorylated on serine, threonine or tyrosine residues and the extent of phosphorylation is regulated by the opposing action of phosphatases, which remove the phosphate moieties. While the majority of protein phosphorylation within the cell is on serine and threonine residues, tyrosine phosphorylation is modulated to the greatest extent during oncogenic transformation and growth factor stimulation (Zhang, *Crit. Rev. Biochem. Mol. Biol.*, **1998**, *33*, 1-52).

Because phosphorylation is such a ubiquitous process within cells and because cellular phenotypes are largely influenced by the activity of these pathways, it is currently believed that a number of disease states and/or disorders are a result of either aberrant activation of, or functional mutations in, kinases and phosphatases. Consequently, considerable attention has been devoted recently to the characterization of tyrosine kinases and tyrosine phosphatases.

PTP1B (also known as protein phosphatase 1B and PTPN1) is an endoplasmic reticulum (ER)-associated enzyme originally isolated as the major protein tyrosine phosphatase of the human placenta (Tonks et al., *J. Biol. Chem.*, **1988**, *263*, 6731-6737; Tonks et al., *J. Biol. Chem.*, **1988**, *263*, 6722-6730).

An essential regulatory role in signaling mediated by the insulin receptor has been established for PTP1B. PTP1B interacts with and dephosphorylates the activated insulin receptor both in vitro and in intact cells resulting in the downregulation of the

- 2 -

signaling pathway (Goldstein et al., *Mol. Cell. Biochem.,* **1998,** *182,* 91-99; Seely et al., *Diabetes,* **1996,** *45,* 1379-1385). In addition, PTP1B modulates the mitogenic actions of insulin (Goldstein et al., *Mol. Cell. Biochem.,* **1998,** *182,* 91-99). In rat adipose cells overexpressing PTP1B, the translocation of the GLUT4 glucose transporter was

5   inhibited, implicating PTP1B as a negative regulator of glucose transport as well (Chen et al., *J. Biol. Chem.,* **1997,** *272,* 8026-8031).

Mouse knockout models lacking the PTP1B gene also point toward the negative regulation of insulin signaling by PTP1B. Mice harboring a disrupted PTP1B gene showed increased insulin sensitivity, increased phosphorylation of the insulin receptor

10   and when placed on a high-fat diet, PTP1B -/- mice were resistant to weight gain and remained insulin sensitive (Elchebly et al., *Science,* **1999,** *283,* 1544-1548). These studies clearly establish PTP1B as a therapeutic target in the treatment of diabetes and obesity.

PTP1B, which is differentially regulated during the cell cycle (Schievella et al.,

15   *Cell. Growth Differ.,* **1993,** *4,* 239-246), is expressed in insulin sensitive tissues as two different isoforms that arise from alternate splicing of the pre-mRNA (Shifrin and Neel, *J. Biol. Chem.,* **1993,** *268,* 25376-25384). It was recently demonstrated that the ratio of the alternatively spliced products is affected by growth factors such as insulin and differs in various tissues examined (Sell and Reese, *Mol. Genet. Metab.,* **1999,** *66,* 189-

20   192). In these studies it was also found that the levels of the variants correlated with the plasma insulin concentration and percentage body fat and may therefore be used as a biomarker for patients with chronic hyperinsulinemia or type 2 diabetes.

Liu and Chernoff have shown that PTP1B binds to and serves as a substrate for the epidermal growth factor receptor (EGFR) (Liu and Chernoff, *Biochem. J.,* **1997,**

25   *327,* 139-145). Furthermore, in A431 human epidermoid carcinoma cells, PT1B was found to be inactivated by the presence of H_2O_2 generated by the addition of EGF. These studies indicate that PTP1B can be negatively regulated by the oxidation state of the cell, which is often deregulated during tumorigenesis (Lee et al., *J. Biol. Chem.,* **1998,** *273,* 15366-15372).

- 3 -

Overexpression of PTP1B has been demonstrated in malignant ovarian cancers
and this correlation was accompanied by a concomitant increase in the expression of
the associated growth factor receptor (Wiener et al., *Am. J. Obstet. Gynecol.*, **1994**,
170, 1177-1183).

5   PTP1B has been shown to suppress transformation in NIH3T3 cells induced by
the neu oncogene (Brown-Shimer et al., *Cancer Res.*, **1992**, *52*, 478-482), as well as in
rat 3Y1 fibroblasts induced by v-srk, v-src, and v-ras (Liu et al., *Mol. Cell. Biol.*, **1998**,
18, 250-259) and rat-1 fibroblasts induced by bcr-abl (LaMontagne et al., *Proc. Natl.
Acad. Sci. U. S. A.*, **1998**, *95*, 14094-14099). It has also been shown that PTP1B
10   promotes differentiation of K562 cells, a chronic myelogenous leukemia cell line, in a
similar manner as does an inhibitor of the bcr-abl oncoprotein. These studies describe
the possible role of PTP1B in controlling the pathogenesis of chronic myeloid leukemia
(LaMontagne et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1998**, *95*, 14094-14099).

PTP1B negatively regulates integrin signaling by interacting with one or more
15   adhesion-dependent signaling components and repressing integrin-mediated MAP
kinase activation (Liu et al., *Curr. Biol.*, **1998**, *8*, 173-176). Other studies designed to
study integrin signaling, using a catalytically inactive form of PTP1B, have shown that
PTP1B regulates cadherin-mediated cell adhesion (Balsamo et al., *J. Cell. Biol.*, **1998**,
143, 523-532) as well as cell spreading, focal adhesion and stress fiber formation and
20   tyrosine phosphorylation (Arregui et al., *J. Cell. Biol.*, **1998**, *143*, 861-873).

Currently, therapeutic agents designed to inhibit the synthesis or action of
PTP1B include small molecules (Ham et al., *Bioorg. Med. Chem. Lett.*, **1999**, *9*, 185-
186; Skorey et al., *J. Biol. Chem.*, **1997**, *272*, 22472-22480; Taing et al., *Biochemistry*,
1999, *38*, 3793-3803; Taylor et al., *Bioorg. Med. Chem.*, **1998**, *6*, 1457-1468; Wang et
25   al., *Bioorg. Med. Chem. Lett.*, **1998**, *8*, 345-350; Wang et al., *Biochem. Pharmacol.*,
1997, *54*, 703-711; Yao et al., *Bioorg. Med. Chem.*, **1998**, *6*, 1799-1810) and peptides
(Chen et al., *Biochemistry*, **1999**, *38*, 384-389; Desmarais et al., *Arch. Biochem.
Biophys.*, **1998**, *354*, 225-231; Roller et al., *Bioorg. Med. Chem. Lett.*, **1998**, *8*, 2149-
2150). In addition, disclosed in the PCT publication WO 97/32595 are
30   phosphopeptides and antibodies that inhibit the association of PTP1B with the activated

- 4 -

insulin receptor for the treatment of disorders associated with insulin resistance. Antisense nucleotides against PTP1B are also generally disclosed (Olefsky, 1997).

There remains a long felt need for additional agents capable of effectively inhibiting PTP1B function and antisense technology is emerging as an effective means for reducing the expression of specific gene products. This technology may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of PTP1B expression.

The present invention, therefore, provides compositions and methods for modulating PTP1B expression, including modulation of the alternatively spliced form of PTP1B.

SUMMARY OF THE INVENTION

The present invention is directed to antisense compounds, particularly oligonucleotides, which are targeted to a nucleic acid encoding PTP1B, and which modulate the expression of PTP1B. Pharmaceutical and other compositions comprising the antisense compounds of the invention are also provided. Further provided are methods of modulating the expression of PTP1B in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of PTP1B by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric antisense compounds, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding PTP1B, ultimately modulating the amount of PTP1B produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding PTP1B. As used herein, the terms "target nucleic acid" and

- 5 -

"nucleic acid encoding PTP1B" encompass DNA encoding PTP1B, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of

5   function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield

10   one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of PTP1B. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the

15   preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene

20   (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding PTP1B. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of

25   expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation

30   codon is also referred to as the "AUG codon," the "start codon" or the "AUG start

- 6 -

codon". A minority of genes have a translation initiation codon having the RNA

sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been

shown to function in vivo. Thus, the terms "translation initiation codon" and "start

codon" can encompass many codon sequences, even though the initiator amino acid in

5   each instance is typically methionine (in eukaryotes) or formylmethionine (in

prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may

have two or more alternative start codons, any one of which may be preferentially

utilized for translation initiation in a particular cell type or tissue, or under a particular

set of conditions. In the context of the invention, "start codon" and "translation

10   initiation codon" refer to the codon or codons that are used in vivo to initiate

translation of an mRNA molecule transcribed from a gene encoding PTP1B, regardless

of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon")

of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the

15   corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The

terms "start codon region" and "translation initiation codon region" refer to a portion of

such an mRNA or gene that encompasses from about 25 to about 50 contiguous

nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon.

Similarly, the terms "stop codon region" and "translation termination codon region"

20   refer to a portion of such an mRNA or gene that encompasses from about 25 to about

50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation

termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to

refer to the region between the translation initiation codon and the translation

25   termination codon, is also a region which may be targeted effectively. Other target

regions include the 5' untranslated region (5'UTR), known in the art to refer to the

portion of an mRNA in the 5' direction from the translation initiation codon, and thus

including nucleotides between the 5' cap site and the translation initiation codon of an

mRNA or corresponding nucleotides on the gene, and the 3' untranslated region

30   (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from

- 7 -

the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding

- 8 -

positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA

5   target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of

10   complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

Antisense compounds are commonly used as research reagents and diagnostics.

15   For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

20   The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be

25   useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring

30   nucleobases, sugars and covalent internucleoside (backbone) linkages as well as

- 9 -

oligonucleotides having non-naturally-occurring portions which function similarly.
Such modified or substituted oligonucleotides are often preferred over native forms
because of desirable properties such as, for example, enhanced cellular uptake,
enhanced affinity for nucleic acid target and increased stability in the presence of

5   nucleases.

      While antisense oligonucleotides are a preferred form of antisense compound,
the present invention comprehends other oligomeric antisense compounds, including
but not limited to oligonucleotide mimetics such as are described below. The antisense
compounds in accordance with this invention preferably comprise from about 8 to

10   about 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly
preferred antisense compounds are antisense oligonucleotides, even more preferably
those comprising from about 12 to about 30 nucleobases. As is known in the art, a
nucleoside is a base-sugar combination. The base portion of the nucleoside is normally
a heterocyclic base. The two most common classes of such heterocyclic bases are the

15   purines and the pyrimidines. Nucleotides are nucleosides that further include a
phosphate group covalently linked to the sugar portion of the nucleoside. For those
nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to
either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the
phosphate groups covalently link adjacent nucleosides to one another to form a linear

20   polymeric compound. In turn the respective ends of this linear polymeric structure can
be further joined to form a circular structure, however, open linear structures are
generally preferred. Within the oligonucleotide structure, the phosphate groups are
commonly referred to as forming the internucleoside backbone of the oligonucleotide.
The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

25   Specific examples of preferred antisense compounds useful in this invention
include oligonucleotides containing modified backbones or non-natural internucleoside
linkages. As defined in this specification, oligonucleotides having modified backbones
include those that retain a phosphorus atom in the backbone and those that do not have
a phosphorus atom in the backbone. For the purposes of this specification, and as

30   sometimes referenced in the art, modified oligonucleotides that do not have a

- 10 -

phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH$_2$ component parts.

- 11 -

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240;

5   5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with

10   novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an

15   aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in

20   Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular -CH_2-NH-O-CH_2-, -CH_2-N(CH_3)-O-CH_2- [known as a methylene (methylimino) or MMI backbone], -CH_2-O-N(CH_3)-CH_2-, -CH_2-N(CH_3)-N(CH_3)-CH_2-

25   and -O-N(CH_3)-CH_2-CH_2- [wherein the native phosphodiester backbone is represented as -O-P-O-CH_2-] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

- 12 -

Modified oligonucleotides may also contain one or more substituted sugar
moieties. Preferred oligonucleotides comprise one of the following at the 2' position:
OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-
alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to
5   C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$,
$O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and
$O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred
oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl,
substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl,
10   Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl,
heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA
cleaving group, a reporter group, an intercalator, a group for improving the
pharmacokinetic properties of an oligonucleotide, or a group for improving the
pharmacodynamic properties of an oligonucleotide, and other substituents having
15   similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-
$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv.
Chim. Acta*, **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred
modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group,
also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-
20   dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl
or 2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-N(CH_2)$_2$, also described in examples
hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy
(2'-O$CH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at
25   other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3'
terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal
nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties
in place of the pentofuranosyl sugar. Representative United States patents that teach
the preparation of such modified sugar structures include, but are not limited to, U.S.:
30   4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786;

- 13 -

5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300;

5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of

which are commonly owned with the instant application, and each of which is herein

incorporated by reference in its entirety.

5   Oligonucleotides may also include nucleobase (often referred to in the art simply

as "base") modifications or substitutions. As used herein, "unmodified" or "natural"

nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine

bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other

synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl

10   cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives

of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-

thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl

uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-

thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted

15   adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-

substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine

and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-

deazaadenine. Further nucleobases include those disclosed in United States Patent No.

3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And*

20   *Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those

disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, *30*, 613,

and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and*

Applications, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**.

Certain of these nucleobases are particularly useful for increasing the binding affinity of

25   the oligomeric compounds of the invention. These include 5-substituted pyrimidines,

6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-

adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have

been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S.,

Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press,

- 14 -

Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem. Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al., *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937.

- 15 -

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603;

5　　5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810;

10　　5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be

15　　incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit,

20　　i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of

25　　cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric

30　　oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides

- 16 -

hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804;

- 17 -

5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, *66*, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt

- 18 -

form with an acid and isolating the free acid in the conventional manner. The free acid
forms differ from their respective salt forms somewhat in certain physical properties
such as solubility in polar solvents, but otherwise the salts are equivalent to their
respective free acid for purposes of the present invention. As used herein, a

5   "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid
form of one of the components of the compositions of the invention. These include
organic or inorganic acid salts of the amines. Preferred acid salts are the
hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable
pharmaceutically acceptable salts are well known to those skilled in the art and include

10   basic salts of a variety of inorganic and organic acids, such as, for example, with
inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid
or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or
N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid,
succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic

15   acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid,
citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic
acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or
isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in
the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also

20   with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid,
2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid,
4-methylbenzenesulfoic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic
acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with
the formation of cyclamates), or with other acid organic compounds, such as ascorbic

25   acid. Pharmaceutically acceptable salts of compounds may also be prepared with a
pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are
well known to those skilled in the art and include alkaline, alkaline earth, ammonium
and quaternary ammonium cations. Carbonates or hydrogen carbonates are also
possible.

- 19 -

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid,

5   hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid,

10   naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be

15   treated by modulating the expression of PTP1B is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically,

20   e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding PTP1B, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid

25   encoding PTP1B can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of PTP1B in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and

30   formulations which include the antisense compounds of the invention. The

- 20 -

. pharmaceutical compositions of the present invention may be administered in a number
of ways depending upon whether local or systemic treatment is desired and upon the
area to be treated. Administration may be topical (including ophthalmic and to mucous
membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or
5   insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal,
epidermal and transdermal), oral or parenteral. Parenteral administration includes
intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or
infusion; or intracranial, e.g., intrathecal or intraventricular, administration.
Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be
10   particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may
include transdermal patches, ointments, lotions, creams, gels, drops, suppositories,
sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder
or oily bases, thickeners and the like may be necessary or desirable. Coated condoms,
15   gloves and the like may also be useful.

Compositions and formulations for oral administration include powders or
granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or
tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders
may be desirable.

20   Compositions and formulations for parenteral, intrathecal or intraventricular
administration may include sterile aqueous solutions which may also contain buffers,
diluents and other suitable additives such as, but not limited to, penetration enhancers,
carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited
25   to, solutions, emulsions, and liposome-containing formulations. These compositions
may be generated from a variety of components that include, but are not limited to,
preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may
conveniently be presented in unit dosage form, may be prepared according to
30   conventional techniques well known in the pharmaceutical industry. Such techniques

- 21 -

include the step of bringing into association the active ingredients with the
pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by
uniformly and intimately bringing into association the active ingredients with liquid
carriers or finely divided solid carriers or both, and then, if necessary, shaping the
product.

The compositions of the present invention may be formulated into any of many
possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft
gels, suppositories, and enemas. The compositions of the present invention may also be
formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous
suspensions may further contain substances which increase the viscosity of the
suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or
dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions
may be formulated and used as foams. Pharmaceutical foams include formulations such
as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While
basically similar in nature these formulations vary in the components and the
consistency of the final product. The preparation of such compositions and
formulations is generally known to those skilled in the pharmaceutical and formulation
arts and may be applied to the formulation of the compositions of the present invention.

Emulsions

The compositions of the present invention may be prepared and formulated as
emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in
another in the form of droplets usually exceeding 0.1 μm in diameter. (Idson, in
Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel
Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage
Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York,
N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger
and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335;
Higuchi *et al.*, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton,

- 22 -

PA, **1985**, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the

5   resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself

10   as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple

15   binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the

20   dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either

25   phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide

30   applicability in the formulation of emulsions and have been reviewed in the literature

- 23 -

(Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.),
1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in
Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), Marcel Dekker,
Inc., New York, N.Y., **1988**, volume 1, p. 199). Surfactants are typically amphiphilic

5   and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to
the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile
balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the
preparation of formulations. Surfactants may be classified into different classes based
on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric

10   (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.),
1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin,
beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic
properties such that they can soak up water to form w/o emulsions yet retain their

15   semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely
divided solids have also been used as good emulsifiers especially in combination with
surfactants and in viscous preparations. These include polar inorganic solids, such as
heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite,
kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum

20   silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion
formulations and contribute to the properties of emulsions. These include fats, oils,
waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids,
preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman,

25   Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p.
335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.),
1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and
synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid,

30   carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for

- 24 -

example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical*

- 25 -

Dosage Forms, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol

5   to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, *in: Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., **1989**, VCH Publishers, New York, pages 185-215). Microemulsions commonly are

10   prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical*

15   *Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 271).

       The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p.

20   245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

25   Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750),

30   decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in

- 26 -

combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may,

however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, 1994, *11*, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, 1994, *11*, 1385; Ho *et al.*, *J. Pharm. Sci.*, 1996, *85*, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of

- 27 -

oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide

- 28 -

range of water and lipid soluble drugs, liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the
5   preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with
10   the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such
15   advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including
20   high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable
25   complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al., Biochem. Biophys. Res. Commun.*, **1987**, *147*, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather
30   than complex with it. Since both the DNA and the lipid are similarly charged, repulsion

- 29 -

rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled*

5   *Release*, **1992**, *19*, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed

10   from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

15   Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (*e.g.* as a solution or as an emulsion) were ineffective (Weiner *et al.*, *Journal of Drug Targeting*, **1992**, *2*, 405-410). Further, an additional study tested the efficacy of

20   interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis *et al.*, *Antiviral Research*, **1992**, *18*, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility

25   in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/ cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic

- 30 -

liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. *S.T.P.Pharma. Sci.*, **1994**, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when
5   incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol
10   (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, *FEBS Letters*, **1987**, *223*, 42; Wu *et al.*,
15   *Cancer Research*, **1993**, *53*, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, **1987**, *507*, 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by
20   Gabizon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*, **1988**, *85*, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-*sn*-dimyristoylphosphatidylcholine are disclosed in WO
25   97/13499 (Lim *et al.*).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (*Bull. Chem. Soc. Jpn.*, **1980**, *53*, 2778) described liposomes comprising a nonionic detergent, $2C_{12}15G$, that contains a PEG moiety. Illum *et al.* (*FEBS Lett.*, **1984**, *167*,
30   79) noted that hydrophilic coating of polystyrene particles with polymeric glycols

- 31 -

results in significantly enhanced blood half-lives. Synthetic phospholipids modified by
the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described
by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (*FEBS Lett.*,
1990, *268*, 235) described experiments demonstrating that liposomes comprising

5   phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant
increases in blood circulation half-lives. Blume *et al.* (*Biochimica et Biophysica Acta*,
1990, *1029*, 91) extended such observations to other PEG-derivatized phospholipids,
e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine
(DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external

10   surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to
Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with
PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Patent Nos.
5,013,556 and 5,356,633) and Martin *et al.* (U.S. Patent No. 5,213,804 and European
Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer

15   conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to
Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-
modified ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Patent Nos.
5,540,935 (Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe PEG-containing
liposomes that can be further derivatized with functional moieties on their surfaces.

20   A limited number of liposomes comprising nucleic acids are known in the art.
WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular
weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa *et al.*
discloses protein-bonded liposomes and asserts that the contents of such liposomes may
include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.* describes

25   certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787
to Love *et al.* discloses liposomes comprising antisense oligonucleotides targeted to the
raf gene.

       Transfersomes are yet another type of liposomes, and are highly deformable lipid
aggregates which are attractive candidates for drug delivery vehicles. Transfersomes

30   may be described as lipid droplets which are so highly deformable that they are easily

- 32 -

able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.* they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to

5   add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including

10   microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms,*

15   Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters

20   such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant

25   class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl

30   benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates.

- 33 -

The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous

- 34 -

solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, *J. Pharm. Pharmacol.*, **1988**, *40*, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-10} alkyl esters thereof (*e.g.*, methyl, isopropyl and *t*-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., **1992**, *44*, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, **1996**, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, *Critical*

- 35 -

Reviews in Therapeutic Drug Carrier Systems, **1991**, page 92; Swinyard, Chapter 39 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, **1990**, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; Yamamoto *et al.*, *J. Pharm. Exp. Ther.*, **1992**, *263*, 25; Yamashita *et al.*, *J. Pharm. Sci.*, **1990**, *79*, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, **1993**, *618*, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)(Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; Buur *et al.*, *J. Control Rel.*, **1990**, *14*, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, **1987**, *39*, 621-626).

- 36 -

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al*, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, **1995**, *5*, 115-121; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, **1996**, *6*, 177-183).

Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically

inert vehicle for delivering one or more nucleic acids to an animal. The excipient may
be liquid or solid and is selected, with the planned manner of administration in mind, so
as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid
and the other components of a given pharmaceutical composition. Typical

5   pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*,
pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose,
etc.); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin,
calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*);
lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid,

10   metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols,
sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch
glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*).

       Pharmaceutically acceptable organic or inorganic excipient suitable for non-
parenteral administration which do not deleteriously react with nucleic acids can also be

15   used to formulate the compositions of the present invention. Suitable pharmaceutically
acceptable carriers include, but are not limited to, water, salt solutions, alcohols,
polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid,
viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

       Formulations for topical administration of nucleic acids may include sterile and

20   non-sterile aqueous solutions, non-aqueous solutions in common solvents such as
alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may
also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable
organic or inorganic excipients suitable for non-parenteral administration which do not
deleteriously react with nucleic acids can be used.

25   Suitable pharmaceutically acceptable excipients include, but are not limited to,
water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose,
magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose,
polyvinylpyrrolidone and the like.

- 38 -

Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., **1987**, Rahway, N.J., pages 1206-1228). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and

- 39 -

ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more

5   combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more

10   combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the

15   disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on $EC_{50}s$ found to be effective in

20   in vitro and in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to

25   have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to

30   illustrate the invention and are not intended to limit the same.

- 40 -

EXAMPLES

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis

Deoxy and 2'-alkoxy amidites

5   2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were

purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research,

Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as

described in U.S. Patent 5,506,351, herein incorporated by reference. For

oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for

10   unmodified oligonucleotides was utilized, except the wait step after pulse delivery of

tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides

were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids*

Research, **1993**, *21*, 3197-3203] using commercially available phosphoramidites (Glen

15   Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites

        2'-Fluorodeoxyadenosine amidites

        2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki,

et. al., *J. Med. Chem.*, **1993**, *36*, 831-841] and United States patent 5,670,633, herein

20   incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-

fluoroadenosine was synthesized utilizing commercially available 9-beta-D-

arabinofuranosyladenine as starting material and by modifying literature procedures

whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-

trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively

25   protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate.

Deprotection of the THP and N6-benzoyl groups was accomplished using standard

methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-

(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

- 41 -

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-

5   arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-

10   phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain

15   the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-

20   3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, *78*, 486-504.

25

- 42 -

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated

5   to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured

10   into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography

15   using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure

20   vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column

25   (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

- 43 -

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with
pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of
dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room
5   temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278
M) was added and the reaction stirred for an additional one hour. Methanol (170 mL)
was then added to stop the reaction. HPLC showed the presence of approximately
70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The
residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated
10     $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4,
filtered and evaporated. 275 g of residue was obtained. The residue was purified on a
3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1)
containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product.
Approximately 20 g additional was obtained from the impure fractions to give a total
15   yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M),
DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of
pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room
20   temperature for 24 hours. The reaction was monitored by TLC by first quenching the
TLC sample with the addition of MeOH. Upon completion of the reaction, as judged
by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue
was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium
bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted
25   with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and
evaporated to give 122 g of residue (approx. 90% product). The residue was purified
on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product
fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered
from later fractions.

- 44 -

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside.

5   Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction

10   mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

15   ### 2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL)

20   and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85

25   g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with

- 45 -

stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl₃ (700 mL) and extracted with saturated NaHCO₃ (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO₄ and

5   evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et₃NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

10   N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH₂Cl₂ (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture

15   was extracted with saturated NaHCO₃ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH₂Cl₂ (300 mL), and the extracts were combined, dried over MgSO₄ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

20   **2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites**

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the

25   following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are

- 46 -

protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416
mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the

- 47 -

reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm

water bath (40-100°C) with the more extreme conditions used to remove the ethylene

glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can

be partitioned between ethyl acetate and water. The product will be in the organic

5   phase.] The residue was purified by column chromatography (2kg silica gel, ethyl

acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined,

stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting

material (17.4g) and pure reusable starting material 20g. The yield based on starting

material less pure recovered starting material was 58%. TLC and NMR were

10   consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g,

36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-

hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P_2O_5 under high

15   vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry

THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-

azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture.

The rate of addition is maintained such that resulting deep red coloration is just

discharged before adding the next drop. After the addition was complete, the reaction

20   was stirred for 4 hrs. By that time TLC showed the completion of the reaction

(ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue

obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to

get 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine as white foam

(21.819 g, 86%).

25   **5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine**

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1g,

4.5mmol) was dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL,

4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered,

- 48 -

the filtrate was washed with ice cold CH₂Cl₂ and the combined organic phase was
washed with water, brine and dried over anhydrous Na₂SO₄. The solution was
concentrated to get 2'-*O*-(aminooxyethyl) thymidine, which was then dissolved in
MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was
5   added and the resulting mixture was strirred for 1 h. Solvent was removed under
vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-
formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

**5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-
methyluridine**

10   5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine
(1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate
(PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was
added to this solution at 10°C under inert atmosphere. The reaction mixture was
stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice
15   bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5%
MeOH in CH₂Cl₂). Aqueous NaHCO₃ solution (5%, 10mL) was added and extracted
with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na₂SO₄,
evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH
(30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction
20   mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to
10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and
reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture
was removed from the ice bath and stirred at room temperature for 2 hrs. To the
reaction mixture 5% NaHCO₃ (25mL) solution was added and extracted with ethyl
25   acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na₂SO₄ and
evaporated to dryness . The residue obtained was purified by flash column
chromatography and eluted with 5% MeOH in CH₂Cl₂ to get 5'-O-*tert*-
butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam
(14.6g, 80%).

- 49 -

2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF
and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of
triethylamine-2HF was then added to 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-
dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room
temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH$_2$Cl$_2$).
Solvent was removed under vacuum and the residue placed on a flash column and
eluted with 10% MeOH in CH$_2$Cl$_2$ to get 2'-O-(dimethylaminooxyethyl)-5-
methyluridine (766mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried
over P$_2$O$_5$ under high vacuum overnight at 40°C. It was then co-evaporated with
anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL)
under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-
dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction
mixture was stirred at room temperature until all of the starting material disappeared.
Pyridine was removed under vacuum and the residue chromatographed and eluted with
10% MeOH in CH$_2$Cl$_2$ (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-
(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08g, 1.67mmol)
was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine
tetrazonide (0.29g, 1.67mmol) was added and dried over P$_2$O$_5$ under high vacuum
overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile
(8.4mL) and 2-cyanoethyl-N,N,N^1,N^1-tetraisopropylphosphoramidite (2.12mL,
6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4
hrs under inert atmosphere. The progress of the reaction was monitored by TLC

(hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous $NaHCO_3$ (40mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-

5   dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following

10   paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

15   The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-

20   ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-

25   diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-

- 51 -

isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-
dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-
O-dimethylaminoethoxyethyl, i.e., $2'\text{-}O\text{-}CH_2\text{-}O\text{-}CH_2\text{-}N(CH_2)_2$, or 2'-DMAEOE
nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared
similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added
to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100
mL bomb. Hydrogen gas evolves as the solid dissolves. $O^2\text{-},2'$-anhydro-5-methyluridine
(1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed,
placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room
temperature and opened. The crude solution is concentrated and the residue
partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is
extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate
(3x200 mL) and the combined organic layers are washed once with water, dried over
anhydrous sodium sulfate and concentrated. The residue is columned on silica gel
using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As
the column fractions are concentrated a colorless solid forms which is collected to give
the title compound as a white solid.

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)   ethyl)]-5-methyl
uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyl
uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl
chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction
mixture is poured into water (200 mL) and extracted with CH_2Cl_2 (2x200 mL). The

- 52 -

combined CH_2Cl_2 layers are washed with saturated $NaHCO_3$ solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using $MeOH:CH_2Cl_2:Et_3N$ (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH_2Cl_2 (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

- 53 -

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

- 54 -

Example 4

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, **1996**, *4*, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness.

- 55 -

Treatment in methanolic ammonia for 24 hrs at room temperature is then done to
deprotect all bases and sample was again lyophilized to dryness. The pellet is
resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2'
positions. The reaction is then quenched with 1M TEAA and the sample is then
reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion
column. The oligo recovered is then analyzed spectrophotometrically for yield and for
purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxyethyl)] chimeric
phosphorothioate oligonucleotides were prepared as per the procedure above for the
2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl)
amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-
(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the
above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of
2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to
generate the phosphodiester internucleotide linkages within the wing portions of the
chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide
(Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the
center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric
oligonucleotides/oligonucleosides are synthesized according to United States patent
5,623,065, herein incorporated by reference.

- 56 -

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the

5   oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance

10   spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7

15   **Oligonucleotide Synthesis - 96 Well Plate Format**

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide

20   linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods.

25   They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH$_4$OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford

- 57 -

a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8
Oligonucleotide Analysis - 96 Well Plate Format

5   The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition
10   was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9
Cell culture and oligonucleotide treatment

         The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following 5 cell types are provided for illustrative
20   purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, Ribonuclease protection assays, or RT-PCR.

T-24 cells:

25   The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies,

- 58 -

Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into
5   96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

10   A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100
15   units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics
20   Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

25   Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as

- 59 -

recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

PC-12 cells:

5   The rat neuronal cell line PC-12 was obtained from the American Type Culure Collection (Manassas, VA). PC-12 cells were routinely cultured in DMEM, high glucose (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% horse serum + 5% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. 10   Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 20000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Treatment with antisense compounds:

15   When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 μL OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 μL of OPTI-MEM™-1 containing 3.75 μg/mL LIPOFECTIN™ (Gibco BRL) and the desired concentration of oligonucleotide. After 4-7 hours of treatment, the medium was 20   replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For 25   human cells the positive control oligonucleotide is ISIS 13920, **TCCGTCATCGCTCCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control oligonucleotide is ISIS

15770, **ATGCATTCTGCCCCCAAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl

gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which

is targeted to both mouse and rat c-raf. The concentration of positive control

oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for

5   ISIS 15770) mRNA is then utilized as the screening concentration for new

oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not

achieved, the lowest concentration of positive control oligonucleotide that results in

60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening

concentration in subsequent experiments for that cell line. If 60% inhibition is not

10   achieved, that particular cell line is deemed as unsuitable for oligonucleotide

transfection experiments.

Example 10

Analysis of oligonucleotide inhibition of PTP1B expression

        Antisense modulation of PTP1B expression can be assayed in a variety of ways

15   known in the art. For example, PTP1B mRNA levels can be quantitated by, e.g.,

Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR

(RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be

performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are

taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*,

20   Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Northern

blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al.,

Current Protocols in Molecular Biology, Volume 1, pp. 4.2.1-4.2.9, John Wiley &

Sons, Inc., **1996**. Real-time quantitative (PCR) can be conveniently accomplished

using the commercially available ABI PRISM™ 7700 Sequence Detection System,

25   available from PE-Applied Biosystems, Foster City, CA and used according to

manufacturer's instructions. Prior to quantitative PCR analysis, primer-probe sets

specific to the target gene being measured are evaluated for their ability to be

"multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target

gene and the internal standard gene GAPDH are amplified concurrently in a single

– 61 –

sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each
dilution is amplified in the presence of primer-probe sets specific for GAPDH only,
target gene only ("single-plexing"), or both (multiplexing). Following PCR
amplification, standard curves of GAPDH and target mRNA signal as a function of

5   dilution are generated from both the single-plexed and multiplexed samples. If both the
slope and correlation coefficient of the GAPDH and target signals generated from the
multiplexed samples fall within 10% of their corresponding values generated from the
single-plexed samples, the primer-probe set specific for that target is deemed as
multiplexable. Other methods of PCR are also known in the art.

10   Protein levels of PTP1B can be quantitated in a variety of ways well known in
the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA
or fluorescence-activated cell sorting (FACS). Antibodies directed to PTP1B can be
identified and obtained from a variety of sources, such as the MSRS catalog of
antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional

15   antibody generation methods. Methods for preparation of polyclonal antisera are
taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*,
Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., **1997**. Preparation of
monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current
Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons,

20   Inc., **1997**.

               Immunoprecipitation methods are standard in the art and can be found at, for
example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp.
10.16.1-10.16.11, John Wiley & Sons, Inc., **1998**. Western blot (immunoblot) analysis
is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current

25   Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons,
Inc., **1997**. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and
can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular
Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., **1991**.

Example 11

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, **1996**, *42*, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for
5   example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μL cold PBS. 60 μL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the
10   plate was gently agitated and then incubated at room temperature for five minutes. 55 μL of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 μL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and
15   then air-dried for 5 minutes. 60 μL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

20   **Example 12**

Total RNA Isolation

Total mRNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed
25   from the cells and each well was washed with 200 μL cold PBS. 100 μL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 μL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and

- 63 -

attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer
RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied
for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY
96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash
5   was then repeated and the vacuum was applied for an additional 10 minutes. The plate
was then removed from the QIAVAC™ manifold and blotted dry on paper towels.
The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube
rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 µL
water into each well, incubating 1 minute, and then applying the vacuum for 30
10   seconds. The elution step was repeated with an additional 60 µL water.

The repetitive pipetting and elution steps may be automated using a QIAGEN
Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on
the culture plate, the plate is transferred to the robot deck where the pipetting, DNase
treatment and elution steps are carried out.

15   **Example 13**

Real-time Quantitative PCR Analysis of PTP1B mRNA Levels

Quantitation of PTP1B mRNA levels was determined by real-time quantitative
PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied
Biosystems, Foster City, CA) according to manufacturer's instructions. This is a
20   closed-tube, non-gel-based, fluorescence detection system which allows high-
throughput quantitation of polymerase chain reaction (PCR) products in real-time. As
opposed to standard PCR, in which amplification products are quantitated after the
PCR is completed, products in real-time quantitative PCR are quantitated as they
accumulate. This is accomplished by including in the PCR reaction an oligonucleotide
25   probe that anneals specifically between the forward and reverse PCR primers, and
contains two fluorescent dyes. A reporter dye (e.g., JOE, FAM, or VIC, obtained from
either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City,
CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained
from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster

- 64 -

City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension
5   phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™
10   7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

        PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA.
15   RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM MgCl$_2$, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNAse inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 µL poly(A) mRNA solution. The RT reaction was carried
20   out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

        Probes and primers to human PTP1B were designed to hybridize to a human
25   PTP1B sequence, using published sequence information (GenBank accession number M31724, incorporated herein as SEQ ID NO:3). For human PTP1B the PCR primers were:
        forward primer: GGAGTTCGAGCAGATCGACAA (SEQ ID NO: 4)
        reverse primer: GGCCACTCTACATGGGAAGTC (SEQ ID NO: 5) and the PCR
30   probe was: FAM-AGCTGGGCGGCCATTTACCAGGAT-TAMRA

- 65 -

(SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the
fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is
the quencher dye. For human GAPDH the PCR primers were:
forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 7)

5   reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 8) and the PCR probe
was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ ID NO: 9) where
JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and
TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

        Probes and primers to rat PTP1B were designed to hybridize to a rat PTP1B

10   sequence, using published sequence information (GenBank accession number M33962,
incorporated herein as SEQ ID NO:10). For rat PTP1B the PCR primers were:
forward primer: CGAGGGTGCAAAGTTCATCAT (SEQ ID NO:11)
reverse primer: CCAGGTCTTCATGGGAAAGCT (SEQ ID NO: 12) and the PCR
probe was: FAM-CGACTCGTCAGTGCAGGATCAGTGGA-TAMRA

15   (SEQ ID NO: 13) where FAM (PE-Applied Biosystems, Foster City, CA) is the
fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is
the quencher dye. For rat GAPDH the PCR primers were:
forward primer: TGTTCTAGAGACAGCCGCATCTT (SEQ ID NO: 14)
reverse primer: CACCGACCTTCACCATCTTGT (SEQ ID NO: 15) and the PCR

20   probe was: 5' JOE-TTGTGCAGTGCCAGCCTCGTCTCA- TAMRA 3' (SEQ ID NO:
16) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter
dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

Example 14

Northern blot analysis of PTP1B mRNA levels

25   Eighteen hours after antisense treatment, cell monolayers were washed twice
with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood,
TX). Total RNA was prepared following manufacturer's recommended protocols.
Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2%
agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO,

- 66 -

Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon
membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary
transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc.,
Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes
5   were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400
(Stratagene, Inc, La Jolla, CA) and then robed using QUICKHYB™ hybridization
solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent
conditions.

To detect human PTP1B, a human PTP1B specific probe was prepared by PCR
10   using the forward primer GGAGTTCGAGCAGATCGACAA (SEQ ID NO: 4) and the
reverse primer GGCCACTCTACATGGGAAGTC (SEQ ID NO: 5). To normalize for
variations in loading and transfer efficiency membranes were stripped and probed for
human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo
Alto, CA).

15   To detect rat PTP1B, a rat PTP1B specific probe was prepared by PCR using
the forward primer CGAGGGTGCAAAGTTCATCAT (SEQ ID NO:11) and the
reverse primer CCAGGTCTTCATGGGAAAGCT (SEQ ID NO: 12). To normalize
for variations in loading and transfer efficiency membranes were stripped and probed
for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo
20   Alto, CA).

Hybridized membranes were visualized and quantitated using a
PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular
Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated
controls.

25   **Example 15**

**Antisense inhibition of human PTP1B expression by chimeric phosphorothioate
oligonucleotides having 2'-MOE wings and a deoxy gap**

In accordance with the present invention, a series of oligonucleotides were
designed to target different regions of the human PTP1B RNA, using published

- 67 -

sequences (GenBank accession number M31724, incorporated herein as SEQ ID NO: 3). The oligonucleotides are shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human PTP1B mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 1

Inhibition of human PTP1B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO
107769	5' UTR	3	1	cttagccccgaggcccgccc	0	17
107770	5' UTR	3	41	ctcggcccactgcgccgtct	58	18
107771	Start Codon	3	74	catgacgggccagggcggct	60	19
107772	Coding	3	113	cccggacttgtcgatctgct	95	20
107773	Coding	3	154	ctggcttcatgtcggatatc	88	21
107774	Coding	3	178	ttggccactctacatgggaa	77	22
107775	Coding	3	223	ggactgacgtctctgtacct	75	23
107776	Coding	3	252	gatgtagtttaatccgacta	82	24
107777	Coding	3	280	ctagcgttgatatagtcatt	29	25
107778	Coding	3	324	gggtaagaatgtaactcctt	86	26
107779	Coding	3	352	tgaccgcatgtgttaggcaa	75	27
107780	Coding	3	381	ttttctgctcccacaccatc	30	28
107781	Coding	3	408	ctctgttgagcatgacgaca	78	29
107782	Coding	3	436	gcgcattttaacgaaccttt	83	30
107783	Coding	3	490	aaatttgtgtcttcaaagat	0	31
107784	Coding	3	519	tgatatcttcagagatcaat	57	32
107785	Coding	3	547	tctagctgtcgcactgtata	74	33

107786	Coding	3	575	agtttcttgggttgtaaggt	33	34
107787	Coding	3	604	gtggtatagtggaaatgtaa	51	35
107788	Coding	3	632	tgattcagggactccaaagt	55	36
107789	Coding	3	661	ttgaaaagaaagttcaagaa	17	37
107790	Coding	3	688	gggctgagtgaccctgactc	61	38
107791	Coding	3	716	gcagtgcaccacaacgggcc	81	39
107792	Coding	3	744	aggttccagacctgccgatg	81	40
107793	Coding	3	772	agcaggaggcaggtatcagc	2	41
107794	Coding	3	799	gaagaagggtctttcctctt	53	42
107795	Coding	3	826	tctaacagcactttcttgat	18	43
107796	Coding	3	853	atcaaccccatccgaaactt	0	44
107797	Coding	3	880	gagaagcgcagctggtcggc	82	45
107798	Coding	3	908	tttggcaccttcgatcacag	62	46
107799	Coding	3	952	agctccttccactgatcctg	70	47
107800	Coding	3	1024	tccaggattcgtttgggtgg	72	48
107801	Coding	3	1052	gaactccctgcatttcccat	68	49
107802	Coding	3	1079	ttccttcacccactggtgat	40	50
107803	Coding	3	1148	gtagggtgcggcatttaagg	0	51
107804	Coding	3	1176	cagtgtcttgactcatgctt	75	52
107805	Coding	3	1222	gcctgggcacctcgaagact	67	53
107806	Coding	3	1268	ctcgtccttctcgggcagtg	37	54
107807	Coding	3	1295	gggcttccagtaactcagtg	73	55
107808	Coding	3	1323	ccgtagccacgcacatgttg	80	56
107809	Coding	3	1351	tagcagaggtaagcgccggc	72	57
107810	Stop Codon	3	1379	ctatgtgttgctgttgaaca	85	58
107811	3' UTR	3	1404	ggaggtggagtggaggaggg	51	59
107812	3' UTR	3	1433	ggctctgcgggcagaggcgg	81	60
107813	3' UTR	3	1460	ccgcggcatgcctgctagtc	84	61
107814	3' UTR	3	1489	tctctacgcggtccggcggc	84	62
107815	3' UTR	3	1533	aagatgggttttagtgcaga	65	63
107816	3' UTR	3	1634	gtactctctttcactctcct	69	64
107817	3' UTR	3	1662	ggccccttccctctgcgccg	59	65
107818	3' UTR	3	1707	ctccaggagggagccctggg	57	66
107819	3' UTR	3	1735	gggctgttggcgtgcgccgc	54	67
107820	3' UTR	3	1783	tttaaataaatatggagtgg	0	68
107821	3' UTR	3	1831	gttcaagaaaatgctagtgc	69	69
107822	3' UTR	3	1884	ttgataaagcccttgatgca	74	70
107823	3' UTR	3	1936	atggcaaagccttccattcc	26	71
107824	3' UTR	3	1973	gtcctccttcccagtactgg	60	72
107825	3' UTR	3	2011	ttacccacaatatcactaaa	39	73
107826	3' UTR	3	2045	attatatattatagcattgt	24	74
107827	3' UTR	3	2080	tcacatcatgtttcttatta	48	75
107828	3' UTR	3	2115	ataacagggaggagaataag	0	76
107829	3' UTR	3	2170	ttacatgcattctaatacac	21	77
107830	3' UTR	3	2223	gatcaaagtttctcatttca	81	78
107831	3' UTR	3	2274	ggtcatgcacaggcaggttg	82	79
107832	3' UTR	3	2309	caacaggcttaggaaccaca	65	80

- 69 -

107833	3' UTR	3	2344	aactgcaccctattgctgag	61	81
107834	3' UTR	3	2380	gtcatgccaggaattagcaa	0	82
107835	3' UTR	3	2413	acaggctgggcctcaccagg	58	83
107836	3' UTR	3	2443	tgagttacagcaagaccctg	44	84
107837	3' UTR	3	2473	gaatatggcttcccataccc	0	85
107838	3' UTR	3	2502	ccctaaatcatgtccagagc	87	86
107839	3' UTR	3	2558	gacttggaatggcggaggct	74	87
107840	3' UTR	3	2587	caaatcacggtctgctcaag	31	88
107841	3' UTR	3	2618	gaagtgtggtttccagcagg	56	89
107842	3' UTR	3	2648	cctaaaggaccgtcacccag	42	90
107843	3' UTR	3	2678	gtgaaccgggacagagacgg	25	91
107844	3' UTR	3	2724	gccccacagggtttgagggt	53	92
107845	3' UTR	3	2755	cctttgcaggaagagtcgtg	75	93
107846	3' UTR	3	2785	aaagccacttaatgtggagg	79	94
107847	3' UTR	3	2844	gtgaaaatgctggcaagaga	86	95
107848	3' UTR	3	2970	tcagaatgcttacagcctgg	61	96

As shown in Table 1, SEQ ID NOs 18, 19, 20, 21, 22, 23, 24, 26, 27, 29, 30, 32, 33, 35, 36, 38, 39, 40, 42, 45, 46, 47, 48, 49, 50, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 69, 70, 72, 73, 75, 78, 79, 80, 81, 83, 84, 86, 87, 89, 90, 92, 93, 94, 95, and 96 demonstrated at least 35% inhibition of human PTP1B expression in this assay and are therefore preferred.

Example 16

Antisense inhibition of rat PTP1B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap.

In accordance with the present invention, a second series of oligonucleotides were designed to target different regions of the rat PTP1B RNA, using published sequences (GenBank accession number M33962, incorporated herein as SEQ ID NO: 10). The oligonucleotides are shown in Table 2. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the

- 70 -

oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were
analyzed for their effect on rat PTP1B mRNA levels by quantitative real-time PCR as
described in other examples herein. Data are averages from two experiments. If
present, "N.D." indicates "no data".

5   Table 2

Inhibition of rat PTP1B mRNA levels by chimeric phosphorothioate oligonucleotides
having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO
111549	5' UTR	10	1	caacctccccagcagcggct	32	97
111550	5' UTR	10	33	tcgaggcccgtcgcccgcca	27	98
111551	5' UTR	10	73	cctcggccgtccgccgcgct	34	99
111552	Coding	10	132	tcgatctgctcgaattcctt	49	100
113669	Coding	10	164	cctggtaaatagccgcccag	36	101
113670	Coding	10	174	tgtcgaatatcctggtaaat	63	102
113671	Coding	10	184	actggcttcatgtcgaatat	58	103
113672	Coding	10	189	aagtcactggcttcatgtcg	40	104
111553	Coding	10	190	gaagtcactggcttcatgtc	27	105
113673	Coding	10	191	ggaagtcactggcttcatgt	54	106
113674	Coding	10	192	gggaagtcactggcttcatg	41	107
113675	Coding	10	193	tgggaagtcactggcttcat	56	108
113676	Coding	10	194	atgggaagtcactggcttca	31	109
113677	Coding	10	195	catgggaagtcactggcttc	59	110
113678	Coding	10	225	tttttgttcttaggaagttt	24	111
111554	Coding	10	228	cggtttttgttcttaggaag	45	112
111555	Coding	10	269	tccgactgtggtcaaaaggg	39	113
113679	Coding	10	273	ttaatccgactgtggtcaaa	45	114
113680	Coding	10	298	atagtcattatcttcctgat	49	115
111556	Coding	10	303	ttgatatagtcattatcttc	29	116
113681	Coding	10	330	gcttcctccatttttatcaa	67	117
111557	Coding	10	359	ggccctgggtgaggatatag	20	118
113682	Coding	10	399	cacaccatctcccagaagtg	29	119
111558	Coding	10	405	tgctcccacaccatctccca	48	120
113683	Coding	10	406	ctgctcccacaccatctccc	51	121
113684	Coding	10	407	tctgctcccacaccatctcc	37	122
113685	Coding	10	408	ttctgctcccacaccatctc	54	123
113686	Coding	10	417	cccctgctcttctgctccca	60	124
111559	Coding	10	438	atgcggttgagcatgaccac	15	125
113687	Coding	10	459	tttaacgagcctttctccat	33	126

113688	Coding	10	492	ttttcttctttctgtggcca	54	127
113689	Coding	10	502	gaccatctctttttcttctt	58	128
111560	Coding	10	540	tcagagatcagtgtcagctt	21	129
113690	Coding	10	550	cttgacatcttcagagatca	64	130
113691	Coding	10	558	taatatgacttgacatcttc	46	131
111561	Coding	10	579	aactccaactgccgtactgt	14	132
111562	Coding	10	611	tctctcgagcctcctgggta	38	133
113692	Coding	10	648	ccaaagtcaggccaggtggt	63	134
111563	Coding	10	654	gggactccaaagtcaggcca	31	135
113693	Coding	10	655	agggactccaaagtcaggcc	50	136
113694	Coding	10	656	cagggactccaaagtcaggc	45	137
113695	Coding	10	657	tcagggactccaaagtcagg	49	138
113696	Coding	10	663	ggtgactcagggactccaaa	34	139
111564	Coding	10	705	cctgactctcggactttgaa	53	140
113697	Coding	10	715	gctgagtgagcctgactctc	57	141
113698	Coding	10	726	ccgtgctctgggctgagtga	48	142
111565	Coding	10	774	aaggtccctgacctgccaat	28	143
111566	Coding	10	819	tctttcctcttgtccatcag	34	144
113699	Coding	10	820	gtctttcctcttgtccatca	41	145
113700	Coding	10	821	ggtctttcctcttgtccatc	66	146
113701	Coding	10	822	gggtctttcctcttgtccat	71	147
113702	Coding	10	852	aacagcacttcttgatgtc	39	148
111567	Coding	10	869	ggaacctgcgcatctccaac	0	149
111568	Coding	10	897	tggtcggccgtctggatgag	29	150
113703	Coding	10	909	gagaagcgcagttggtcggc	48	151
113704	Coding	10	915	aggtaggagaagcgcagttg	31	152
113705	Coding	10	918	gccaggtaggagaagcgcag	41	153
111569	Coding	10	919	agccaggtaggagaagcgca	56	154
113706	Coding	10	920	cagccaggtaggagaagcgc	58	155
113707	Coding	10	921	acagccaggtaggagaagcg	43	156
113708	Coding	10	922	cacagccaggtaggagaagc	49	157
113709	Coding	10	923	tcacagccaggtaggagaag	47	158
111570	Coding	10	924	atcacagccaggtaggagaa	51	159
113710	Coding	10	925	gatcacagccaggtaggaga	51	160
113711	Coding	10	926	cgatcacagccaggtaggag	63	161
113712	Coding	10	927	tcgatcacagccaggtagga	71	162
113713	Coding	10	932	caccctcgatcacagccagg	75	163
113714	Coding	10	978	tccttccactgatcctgcac	97	164
111571	Coding	10	979	ctccttccactgatcctgca	89	165
113715	Coding	10	980	gctccttccactgatcctgc	99	166
107799	Coding	10	981	agctccttccactgatcctg	99	167
113716	Coding	10	982	aagctccttccactgatcct	97	168
113717	Coding	10	983	aaagctccttccactgatcc	95	169
113718	Coding	10	984	gaaagctccttccactgatc	95	170
113719	Coding	10	985	ggaaagctccttccactgat	95	171
111572	Coding	10	986	gggaaagctccttccactga	89	172
113720	Coding	10	987	tgggaaagctccttccactg	97	173
113721	Coding	10	1036	tggccggggaggtgggggca	20	174

111573	Coding	10	1040	tgggtggccggggaggtggg	20	175
113722	Coding	10	1046	tgcgtttgggtggccggggga	18	176
111574	Coding	10	1073	tgcacttgccattgtgaggc	38	177
113723	Coding	10	1206	acttcagtgtcttgactcat	67	178
113724	Coding	10	1207	aacttcagtgtcttgactca	60	179
111575	Coding	10	1208	taacttcagtgtcttgactc	50	180
113725	Coding	10	1209	ctaacttcagtgtcttgact	53	181
111576	Coding	10	1255	gacagatgcctgagcacttt	32	182
106409	Coding	10	1333	gaccaggaagggcttccagt	32	183
113726	Coding	10	1334	tgaccaggaagggcttccag	39	184
111577	Coding	10	1335	ttgaccaggaagggcttcca	32	185
113727	Coding	10	1336	gttgaccaggaagggcttcc	41	186
113728	Coding	10	1342	gcacacgttgaccaggaagg	59	187
111578	Coding	10	1375	gaggtacgcgccagtcgcca	45	188
111579	Coding	10	1387	tacccggtaacagaggtacg	32	189
111580	Coding	10	1397	agtgaaaacatacccggtaa	30	190
111581	3' UTR	10	1456	caaatcctaacctgggcagt	31	191
111582	3' UTR	10	1519	ttccagttccaccacaggct	24	192
111583	3' UTR	10	1552	ccagtgcacagatgcccctc	47	193
111584	3' UTR	10	1609	acaggttaaggccctgagat	29	194
111585	3' UTR	10	1783	gcctagcatcttttgttttc	43	195
111586	3' UTR	10	1890	aagccagcaggaactttaca	36	196
111587	3' UTR	10	2002	gggacacctgagggaagcag	16	197
111588	3' UTR	10	2048	ggtcatctgcaagatggcgg	40	198
111589	3' UTR	10	2118	gccaacctctgatgaccctg	25	199
111590	3' UTR	10	2143	tggaagccccagctctaagc	25	200
111591	3' UTR	10	2165	tagtaatgactttccaatca	44	201
111592	3' UTR	10	2208	tgagtcttgctttacacctc	41	202
111593	3' UTR	10	2252	cctgcgcgcggagtgacttc	22	203
111594	3' UTR	10	2299	aggacgtcactgcagcagga	43	204
111595	3' UTR	10	2346	tcaggacaagtcttggcagt	32	205
111596	3' UTR	10	2405	gaggctgcacagtaagcgct	34	206
111597	3' UTR	10	2422	tcagccaaccagcatcagag	20	207
111598	3' UTR	10	2449	acccacagtgtccacctccc	30	208
111599	3' UTR	10	2502	agtgcgggctgtgctgctgg	30	209
111600	3' UTR	10	2553	cagctcgctctggcggcctc	8	210
111601	3' UTR	10	2608	aggaagggagctgcacgtcc	32	211
111602	3' UTR	10	2664	ccctcacgattgctcgtggg	24	212
111603	3' UTR	10	2756	cagtggagcggctcctctgg	18	213
111604	3' UTR	10	2830	caggctgacacacttacacgg	30	214
111605	3' UTR	10	2883	gtcctacctcaaccctagga	37	215
111606	3' UTR	10	2917	ctgccccagcaccagccaca	12	216
111607	3' UTR	10	2946	attgcttctaagaccctcag	33	217
111608	3' UTR	10	2978	ttacatgtcaccactgttgt	28	218
111609	3' UTR	10	3007	tacacatgtcatcagtagcc	37	219
111610	3' UTR	10	3080	ttttctaactcacagggaaa	30	220
111611	3' UTR	10	3153	gtgcccgccagtgagcaggc	23	221
111612	3' UTR	10	3206	cggcctcggcactggacagc	27	222

111613	3' UTR	10	3277	gtggaatgtctgagatccag	31	223
111614	3' UTR	10	3322	agggcgggcctgcttgccca	23	224
111615	3' UTR	10	3384	cggtcctggcctgctccaga	31	225
111616	3' UTR	10	3428	tacactgttcccaggagggt	42	226
111617	3' UTR	10	3471	tggtgccagcagcgctagca	10	227
111618	3' UTR	10	3516	cagtctcttcagcctcaaga	43	228
113729	3' UTR	10	3537	aagagtcatgagcaccatca	56	229
111619	3' UTR	10	3560	tgaaggtcaagttcccctca	40	230
111620	3' UTR	10	3622	ctggcaagaggcagactgga	30	231
111621	3' UTR	10	3666	ggctctgtgctggcttctct	52	232
111622	3' UTR	10	3711	gccatctcctcagcctgtgc	39	233
111623	3' UTR	10	3787	agcgcctgctctgaggcccc	16	234
111624	3' UTR	10	3854	tgctgagtaagtattgactt	35	235
111625	3' UTR	10	3927	ctatggccatttagagagag	36	236
113730	3' UTR	10	3936	tggtttattctatggccatt	59	237
111626	3' UTR	10	3994	cgctcctgcaaaggtgctat	11	238
111627	3' UTR	10	4053	gttggaaacggtgcagtcgg	39	239
111628	3' UTR	10	4095	atttattgttgcaactaatg	33	240

As shown in Table 2, SEQ ID NOs 97, 99, 100, 101, 102, 103, 104, 106, 107, 108, 109, 110, 112, 113, 114, 115, 117, 120, 121, 122, 123, 124, 126, 127, 128, 130, 131, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 144, 145, 146, 147, 148, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 191, 193, 195, 196, 198, 201, 202, 204, 205, 206, 211, 215, 217, 219, 223, 225, 226, 228, 229, 230, 232, 233, 235, 236, 237, 239 and 240 demonstrated at least 30% inhibition of rat PTP1B expression in this experiment and are therefore preferred.

Example 17

Western blot analysis of PTP1B protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to PTP1B is used, with a radiolabelled or fluorescently labeled secondary antibody directed against

– 74 –

the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

Example 18

Effects of antisense inhibition of PTP1B (ISIS 113715) on blood glucose levels

db/db mice are used as a model of Type 2 diabetes. These mice are hyperglycemic, obese, hyperlipidemic, and insulin resistant. The db/db phenotype is due to a mutation in the leptin receptor on a C57BLKS background. However, a mutation in the leptin gene on a different mouse background can produce obesity without diabetes (ob/ob mice). Leptin is a hormone produced by fat that regulates appetite and animals or humans with leptin deficiencies become obese. Heterozygous db/wt mice (known as lean littermates) do not display the hyperglycemia/hyperlipidemia or obesity phenotype and are used as controls.

In accordance with the present invention, ISIS 113715 (GCTCCTTCCACTGATCCTGC, SEQ ID No: 166) was investigated in experiments designed to address the role of PTP1B in glucose metabolism and homeostasis. ISIS 113715 is completely complementary to sequences in the coding region of the human, rat, and mouse PTP1B nucleotide sequences incorporated herein as SEQ ID No: 3 (starting at nucleotide 951 of human PTP1B; Genbank Accession No. M31724), SEQ ID No: 10 (starting at nucleotide 980 of rat PTP1B; Genbank Accession No. M33962) and SEQ ID No: 241 (starting at nucleotide 1570 of mouse PTP1B; Genbank Accession No. U24700). The control used is ISIS 29848 (NNNNNNNNNNNNNNNNNNNN, SEQ ID No: 242) where N is a mixture of A, G, T and C.

Male db/db mice and lean (heterozygous, i.e., db/wt) littermates (age 9 weeks at time 0) were divided into matched groups (n=6) with the same average blood glucose levels and treated by intraperitoneal injection once a week with saline, ISIS 29848 (the control oligonucleotide) or ISIS 113715. db/db mice were treated at a dose of 10, 25 or 50 mg/kg of ISIS 113715 or 50 mg/kg of ISIS 29848 while lean littermates were treated at a dose of 50 or 100 mg/kg of ISIS 113715 or 100 mg/kg of ISIS 29848.

- 75 -

Treatment was continued for 4 weeks with blood glucose levels being measured on day 0, 7, 14, 21 and 28.

By day 28 in db/db mice, blood glucose levels were reduced at all doses from a starting level of 300 mg/dL to 225 mg/dL for the 10 mg/kg dose, 175 mg/dL for the 25 mg/kg dose and 125 mg/dL for the 50 mg/kg dose. These final levels are within normal range for wild-type mice (170 mg/dL). The mismatch control and saline treated levels levels were 320 mg/dL and 370 mg/dL at day 28, respectively.

In lean littermates, blood glucose levels remained constant throughout the study for all treatment groups (average 120 mg/dL). These results indicate that treatment with ISIS 113715 reduces blood glucose in db/db mice and that there is no hypoglycemia induced in the db/db or the lean littermate mice as a result of the oligonucleotide treatment.

In a similar experiment, ob/ob mice and their lean littermates (heterozygous, i.e., ob/wt) were dosed twice a week at 50 mg/kg with ISIS 113715, ISIS 29848 or saline control and blood glucose levels were measured at the end of day 7, 14 and 21. Treatment of ob/ob mice with ISIS 113715 resulted in the largest decrease in blood glucose over time going from 225 mg/dL at day 7 to 95 mg/dL at day 21. Ob/ob mice displayed an increase in plasma glucose over time from 300 mg/dL to 325 mg/dL while treatment with the control oligonucleotide reduced plasma glucose from an average of 280 mg/dL to 130 mg/dL. In the lean littermates plasma glucose levels remained unchanged in all treatment groups (average level 100 mg/dL).

Example 19

Effects of antisense inhibition of PTP1B (ISIS 113715) on mRNA expression in liver

Male db/db mice and lean littermates (age 9 weeks at time 0) were divided into matched groups (n=6) with the same average blood glucose levels and treated by intraperitoneal injection once a week with saline, ISIS 29848 (the control oligonucleotide) or ISIS 113715. db/db mice were treated at a dose of 10, 25 or 50 mg/kg of ISIS 113715 or 50 mg/kg of ISIS 29848 while lean littermates were treated

- 76 -

at a dose of 50 or 100 mg/kg of ISIS 113715 or 100 mg/kg of ISIS 29848. Treatment
was continued for 4 weeks after which the mice were sacrificed and tissues collected
for mRNA analysis. RNA values were normalized and are expressed as a percentage of
saline treated control.

5   ISIS 113715 successfully reduced PTP1B mRNA levels in the livers of db/db
mice at all doses examined (60% reduction of PTP1B mRNA), whereas the control
oligonucleotide treated animals showed no reduction in PTP1B mRNA, remaining at
the level of the saline treated control. Treatment of lean littermates with ISIS 113715
also reduced mRNA levels to 45% of control at the 50 mg/kg dose and 25% of control

10   at the 100 mg/kg dose. The control oligonucleotide (ISIS 29848) failed to show any
reduction in mRNA levels.

Example 20

Effects of antisense inhibition of PTP1B (ISIS 113715) on body weight

               Male db/db mice and lean littermates (age 9 weeks at time 0) were divided into

15   matched groups (n=6) with the same average blood glucose levels and treated by
intraperitoneal injection once a week with saline, ISIS 29848 (the control
oligonucleotide) or ISIS 113715. db/db mice were treated at a dose of 10, 25 or 50
mg/kg of ISIS 113715 or 50 mg/kg of ISIS 29848 while lean littermates were treated
at a dose of 50 or 100 mg/kg of ISIS 113715 or 100 mg/kg of ISIS 29848. Treatment

20   was continued for 4 weeks. At day 28 mice were sacrificed and final body weights
were measured.

               Treatment of ob/ob mice with ISIS 113715 resulted in an increase in body
weight which was constant over the dose range with animals gaining an average of 11.0
grams while saline treated controls gained 5.5 grams. Animals treated with the control

25   oligonucleotide gained an average of 7.8 grams of body weight.

               Lean littermate animals treated with 50 or 100 mg/kg of ISIS 113715 gained
3.8 grams of body weight compared to a gain of 3.0 grams for the saline controls.

- 77 -

In a similar experiment, ob/ob mice and their lean littermates were dosed twice a week at 50 mg/kg with ISIS 113715, ISIS 29848 or saline control and body weights were measured at the end of day 7, 14 and 21.

Treatment of the ob/ob mice with ISIS 113715, ISIS 29848 or saline control all 5 resulted in a similar increase in body weight across the 21-day timecourse. At the end of day 7 all ob/ob treatment groups had an average weight of 42 grams. By day 21, animals treated with ISIS 113715 had an average body weight of 48 grams, while those in the ISIS 29848 (control oligonucleotide) and saline control group each had an average body weight of 52 grams. All of the lean littermates had an average body 10 weight of 25 grams at the beginning of the timecourse and all lean littermate treatment groups showed an increase in body weight, to 28 grams, by day 21.

Example 21

Effects of antisense inhibition of PTP1B (ISIS 113715) on plasma insulin levels

Male db/db mice (age 9 weeks at time 0) were divided into matched groups 15 (n=6) with the same average blood glucose levels and treated by intraperitoneal injection twice a week with saline, ISIS 29848 (the control oligonucleotide) or ISIS 113715 at a dose of 50 mg/kg. Treatment was continued for 3 weeks with plasma insulin levels being measured on day 7, 14, and 21.

Mice treated with ISIS 113715 showed a decrease in plasma insulin levels from 20 15 ng/mL at day 7 to 7.5 ng/mL on day 21. Saline treated animals has plasma insulin levels of 37 ng/mL at day 7 which dropped to 25 ng/mL on day 14 but rose again to 33 ng/mL by day 21. Mice treated with the control oligonucleotide also showed a decrease in plasma insulin levels across the timecourse of the study from 25 ng/mL at day 7 to 10 ng/mL on day 21. However, ISIS 113715 was the most effective at 25 reducing plasma insulin over time.

- 78 -

What is claimed is:

1.   A compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding PTP1B, wherein said compound specifically hybridizes with and inhibits the expression of PTP1B.

2.   The compound of claim 1 which is an antisense oligonucleotide.

3.   The compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 18, 19, 20, 21, 22, 23, 24, 26, 27, 29, 30, 32, 33, 35, 36, 38, 39, 40, 42, 45, 46, 47, 48, 49, 50, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 69, 70, 72, 73, 75, 78, 79, 80, 81, 83, 84, 86, 87, 89, 90, 92, 93, 94, 95, 96, 97, 99, 100, 101, 102, 103, 104, 106, 107, 108, 109, 110, 112, 113, 114, 115, 117, 120, 121, 122, 123, 124, 126, 127, 128, 130, 131, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 144, 145, 146, 147, 148, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 168, 169, 170, 171, 172, 173, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 191, 193, 195, 196, 198, 201, 202, 204, 205, 206, 211, 215, 217, 219, 223, 225, 226, 228, 229, 230, 232, 233, 235, 236, 237, 239 or 240.

4.   The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.

5.   The compound of claim 4 wherein the modified internucleoside linkage is a phosphorothioate linkage.

6.   The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.

7.   The compound of claim 6 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

8.   The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.

9.   The compound of claim 8 wherein the modified nucleobase is a 5-methylcytosine.

10.   The compound of claim 2 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

11.   A composition comprising the compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

12.   The composition of claim 11 further comprising a colloidal dispersion system.

13.   The composition of claim 11 wherein the compound is an antisense oligonucleotide.

14.   A compound 8 to 50 nucleobases in length which specifically hybridizes with at least an 8-nucleobase portion of an active site on a nucleic acid molecule encoding PTP1B.

15.   A method of inhibiting the expression of PTP1B in cells or tissues comprising contacting said cells or tissues with the compound of claim 1 so that expression of PTP1B is inhibited.

- 80 -

16.   The method of claim 15 wherein the cells or tissues are human cells or tissues.

17.   The method of claim 15 wherein the cells or tissues are rodent cells or tissues.

18.   The method of claim 17 wherein the rodent cells or tissues are mouse cells or tissues.

19.   The method of claim 17 wherein the rodent cells or tissues are rat cells or tissues.

20.   The method of claim 15 wherein the cells or tissues are liver, kidney or adipose cells or tissues.

21.   A method of treating an animal having or suspected of having a disease or condition associated with PTP1B comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of PTP1B is inhibited.

22.   The method of claim 21 wherein the animal is a human.

23.   The method of claim 21 wherein the disease or condition is a metabolic disease or condition.

24.   The method of claim 21 wherein the disease or condition is diabetes.

25.   The method of claim 21 wherein the disease or condition is Type 2 diabetes.

- 81 -

26.   The method of claim 21 wherein the disease or condition is obesity.

27.   The method of claim 21 wherein the disease or condition is a hyperproliferative condition.

28.   The method of claim 27 wherein the hyperproliferative condition is cancer.

29.   A method of decreasing blood glucose levels in an animal comprising administering to said animal the compound of claim 1.

30.   The method of claim 29 wherein the animal is a human or a rodent.

31.   The method of claim 29 wherein the blood glucose levels are plasma glucose levels or serum glucose levels.

32.   The method of claim 29 wherein the animal is a diabetic animal.

33.   A method of preventing or delaying the onset of a disease or condition associated with PTP1B in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1.

34.   The method of claim 33 wherein the animal is a human.

35.   The method of claim 33 wherein the disease or condition is a metabolic disease or condition.

36.   The method of claim 33 wherein the disease or condition is diabetes.

37. The method of claim 33 wherein the disease or condition is Type 2 diabetes.

38. The method of claim 33 wherein the disease or condition is obesity.

39. The method of claim 33 wherein the disease or condition is a hyperproliferative condition.

40. The method of claim 39 wherein the hyperproliferative condition is cancer.

41. A method of preventing or delaying the onset of an increase in blood glucose levels in an animal comprising administering to said animal the compound of claim 1.

42. The method of claim 41 wherein the animal is a human or a rodent.

43. The method of claim 41 wherein the blood glucose levels are plasma glucose levels or serum glucose levels.

44. The method of claim 41 wherein the animal is a diabetic animal.

SEQUENCE LISTING

<110> Lex M. Cowsert
      Jacqueline Wyatt
      Susan M. Freier
      Brett P. Monia
      Madeline M. Butler
      Robert McKay

<120> ANTISENSE MODULATION OF PTP1B EXPRESSION

<130> ISPH-0478

<140> US 09/629,644
<141> 2000-07-31

<150> US 09/487,368
<151> 2000-01-18

<160> 242

<210> 1
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 1
tccgtcatcg ctcctcaggg   20

<210> 2
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 2
atgcattctg cccccaagga   20

<210> 3
<211> 3247
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (91)...(1398)

<400> 3
gggcgggcct cggggctaag agcgcgacgc ctagagcggc agacggcgca gtgggccgag   60

aaggaggcgc agcagccgcc ctggcccgtc atg gag atg gaa aag gag ttc gag   114
                                   Met Glu Met Glu Lys Glu Phe Glu

```
                                              1                 5
cag atc gac aag tcc ggg agc tgg gcg gcc att tac cag gat atc cga    . 162
Gln Ile Asp Lys Ser Gly Ser Trp Ala Ala Ile Tyr Gln Asp Ile Arg
      10              15              20

cat gaa gcc agt gac ttc cca tgt aga gtg gcc aag ctt cct aag aac      210
His Glu Ala Ser Asp Phe Pro Cys Arg Val Ala Lys Leu Pro Lys Asn
 ·25              30              35              40

aaa aac cga aat agg tac aga gac gtc agt ccc ttt gac cat agt cgg      258
Lys Asn Arg Asn Arg Tyr Arg Asp Val Ser Pro Phe Asp His Ser Arg
              45              50              55

att aaa cta cat caa gaa gat aat gac tat atc aac gct agt ttg ata      306
Ile Lys Leu His Gln Glu Asp Asn Asp Tyr Ile Asn Ala Ser Leu Ile
          60              65              70

aaa atg gaa gaa gcc caa agg agt tac att ctt acc cag ggc cct ttg   .  354
Lys Met Glu Glu Ala Gln Arg Ser Tyr Ile Leu Thr Gln Gly Pro Leu
      75              80              85

cct aac aca tgc ggt cac ttt tgg gag atg gtg tgg gag cag aaa agc      402
Pro Asn Thr Cys Gly His Phe Trp Glu Met Val Trp Glu Gln Lys Ser
      90              95              100

agg ggt gtc gtc atg ctc aac aga gtg atg gag aaa ggt tcg tta aaa      450
Arg Gly Val Val Met Leu Asn Arg Val Met Glu Lys Gly Ser Leu Lys
105              110             115             120

tgc gca caa tac tgg cca caa aaa gaa gaa aaa gag atg atc ttt gaa      498
Cys Ala Gln Tyr Trp Pro Gln Lys Glu Glu Lys Glu Met Ile Phe Glu
              125             130             135

gac aca aat ttg aaa tta aca ttg atc tct gaa gat atc aag tca tat      546
Asp Thr Asn Leu Lys Leu Thr Leu Ile Ser Glu Asp Ile Lys Ser Tyr
              140             145             150

tat aca gtg cga cag cta gaa ttg gaa aac ctt aca acc caa gaa act      594
Tyr Thr Val Arg Gln Leu Glu Leu Glu Asn Leu Thr Thr Gln Glu Thr
              155             160             165

cga gag atc tta cat ttc cac tat acc aca tgg cct gac ttt gga gtc      642
Arg Glu Ile Leu His Phe His Tyr Thr Thr Trp Pro Asp Phe Gly Val
      170             175             180

cct gaa tca cca gcc tca ttc ttg aac ttt ctt ttc aaa gtc cga gag      690
Pro Glu Ser Pro Ala Ser Phe Leu Asn Phe Leu Phe Lys Val Arg Glu
185             190             195             200

tca ggg tca ctc agc ccg gag cac ggg ccc gtt gtg gtg cac tgc agt      738
Ser Gly Ser Leu Ser Pro Glu His Gly Pro Val Val Val His Cys Ser
              205             210             215

gca ggc atc ggc agg tct gga acc ttc tgt ctg gct gat acc tgc ctc      786
Ala Gly Ile Gly Arg Ser Gly Thr Phe Cys Leu Ala Asp Thr Cys Leu
              220             225             230

ctg ctg atg gac aag agg aaa gac cct tct tcc gtt gat atc aag aaa      834
```

```
        Leu Leu Met Asp Lys Arg Lys Asp Pro Ser Ser Val Asp Ile Lys Lys
                235             240             245

gtg ctg tta gaa atg agg aag ttt cgg atg ggg ttg atc cag aca gcc    882
Val Leu Leu Glu Met Arg Lys Phe Arg Met Gly Leu Ile Gln Thr Ala
        250             255             260

gac cag ctg cgc ttc tcc tac ctg gct gtg atc gaa ggt gcc aaa ttc    930
Asp Gln Leu Arg Phe Ser Tyr Leu Ala Val Ile Glu Gly Ala Lys Phe
265             270             275             280

atc atg ggg gac tct tcc gtg cag gat cag tgg aag gag ctt tcc cac    978
Ile Met Gly Asp Ser Ser Val Gln Asp Gln Trp Lys Glu Leu Ser His
                285             290             295

gag gac ctg gag ccc cca ccc gag cat atc ccc cca cct ccc cgg cca   1026
Glu Asp Leu Glu Pro Pro Pro Glu His Ile Pro Pro Pro Pro Arg Pro
            300             305             310

ccc aaa cga atc ctg gag cca cac aat ggg aaa tgc agg gag ttc ttc   1074
Pro Lys Arg Ile Leu Glu Pro His Asn Gly Lys Cys Arg Glu Phe Phe
            315             320             325

cca aat cac cag tgg gtg aag gaa gag acc cag gag gat aaa gac tgc   1122
Pro Asn His Gln Trp Val Lys Glu Glu Thr Gln Glu Asp Lys Asp Cys
        330             335             340

ccc atc aag gaa gaa aaa gga agc ccc tta aat gcc gca ccc tac ggc   1170
Pro Ile Lys Glu Glu Lys Gly Ser Pro Leu Asn Ala Ala Pro Tyr Gly
345             350             355             360

atc gaa agc atg agt caa gac act gaa gtt aga agt cgg gtc gtg ggg   1218
Ile Glu Ser Met Ser Gln Asp Thr Glu Val Arg Ser Arg Val Val Gly
                365             370             375

gga agt ctt cga ggt gcc cag gct gcc tcc cca gcc aaa ggg gag ccg   1266
Gly Ser Leu Arg Gly Ala Gln Ala Ala Ser Pro Ala Lys Gly Glu Pro
            380             385             390

tca ctg ccc gag aag gac gag gac cat gca ctg agt tac tgg aag ccc   1314
Ser Leu Pro Glu Lys Asp Glu Asp His Ala Leu Ser Tyr Trp Lys Pro
            395             400             405

ttc ctg gtc aac atg tgc gtg gct acg gtc ctc acg gcc ggc gct tac   1362
Phe Leu Val Asn Met Cys Val Ala Thr Val Leu Thr Ala Gly Ala Tyr
            410             415             420

ctc tgc tac agg ttc ctg ttc aac agc aac aca tag cctgaccctc        1408
Leu Cys Tyr Arg Phe Leu Phe Asn Ser Asn Thr
425             430             435

ctccactcca cctccaccca ctgtccgcct ctgcccgcag agcccacgcc cgactagcag   1468

gcatgccgcg gtaggtaagg gccgccggac cgcgtagaga gccgggcccc ggacggacgt   1528

tggttctgca ctaaaaccca tcttccccgg atgtgtgtct cacccctcat ccttttactt   1588

tttgcccctt ccactttgag taccaaatcc acaagccatt ttttgaggag agtgaaagag   1648
```

3

```
agtaccatgc tggcggcgca gagggaaggg gcctacaccc gtcttggggc tcgccccacc   1708
cagggctccc tcctggagca tcccaggcgg cgcacgccaa cagccccccc cttgaatctg   1768
cagggagcaa ctctccactc catatttatt taaacaattt tttccccaaa ggcatccata   1828
gtgcactagc attttcttga accaataatg tattaaaatt ttttgatgtc agccttgcat   1888
caagggcttt atcaaaaagt acaataataa atcctcaggt agtactggga atggaaggct   1948
ttgccatggg cctgctgcgt cagaccagta ctgggaagga ggacggttgt aagcagttgt   2008
tatttagtga tattgtgggt aacgtgagaa gatagaacaa tgctataata tataatgaac   2068
acgtgggtat ttaataagaa acatgatgtg agattacttt gtcccgctta ttctcctccc   2128
tgttatctgc tagatctagt tctcaatcac tgctcccccg tgtgtattag aatgcatgta   2188
aggtcttctt gtgtcctgat gaaaaatatg tgcttgaaat gagaaacttt gatctctgct   2248
tactaatgtg ccccatgtcc aagtccaacc tgcctgtgca tgacctgatc attacatggc   2308
tgtggttcct aagcctgttg ctgaagtcat tgtcgctcag caatagggtg cagttttcca   2368
ggaataggca tttgctaatt cctggcatga cactctagtg acttcctggt gaggcccagc   2428
ctgtcctggt acagcagggt cttgctgtaa ctcagacatt ccaagggtat gggaagccat   2488
attcacacct cacgctctgg acatgattta gggaagcagg gacaccccc gcccccacc    2548
tttgggatca gcctccgcca ttccaagtca acactcttct tgagcagacc gtgatttgga   2608
agagaggcac ctgctggaaa ccacacttct tgaaacagcc tgggtgacgg tcctttaggc   2668
agcctgccgc cgtctctgtc ccggttcacc ttgccgagag aggcgcgtct gccccaccct   2728
caaaccctgt ggggcctgat ggtgctcacg actcttcctg caaagggaac tgaagacctc   2788
cacattaagt ggcttttaa catgaaaaac acggcagctg tagctcccga gctactctct    2848
tgccagcatt ttcacatttt gcctttctcg tggtagaagc cagtacagag aaattctgtg    2908
gtgggaacat tcgaggtgtc accctgcaga gctatggtga ggtgtggata aggcttaggt   2968
gccaggctgt aagcattctg agctggcttg ttgtttttaa gtcctgtata tgtatgtagt   3028
agtttgggtg tgtatatata gtagcatttc aaaatggacg tactggttta acctcctatc   3088
cttggagagc agctggctct ccaccttgtt acacattatg ttagagaggt agcgagctgc   3148
tctgctatat gccttaagcc aatatttact catcaggtca ttattttta caatggccat    3208
ggaataaacc atttttacaa aaataaaaac aaaaaaagc                          3247
```

<210> 4
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 4
ggagttcgag cagatcgaca a   21

<210> 5
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 5
ggccactcta catgggaagt c   21

<210> 6
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Probe

<400> 6
agctgggcgg ccatttacca ggat   24

<210> 7
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 7
gaaggtgaag gtcggagtc   19

<210> 8
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 8
gaagatggtg atgggatttc   20

<210> 9
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Probe

<400> 9
caagcttccc gttctcagcc   20

<210> 10
<211> 4127
<212> DNA
<213> Rattus norvegicus

<220>
<221> CDS
<222> (120)...(1418)

<400> 10
agccgctgct ggggaggttg gggctgaggt ggtggcgggc gacgggcctc gagacgcgga   60

gcgacgcggc ctagcgcggc ggacggccga gggaactcgg gcagtcgtcc cgtcccgcc   119

atg gaa atg gag aag gaa ttc gag cag atc gat aag gct ggg aac tgg   167
Met Glu Met Glu Lys Glu Phe Glu Gln Ile Asp Lys Ala Gly Asn Trp
 1   5   10   15

gcg gct att tac cag gat att cga cat gaa gcc agt gac ttc cca tgc   215
Ala Ala Ile Tyr Gln Asp Ile Arg His Glu Ala Ser Asp Phe Pro Cys
                20   25   30

aga ata gcg aaa ctt cct aag aac aaa aac cgg aac agg tac cga gat   263
Arg Ile Ala Lys Leu Pro Lys Asn Lys Asn Arg Asn Arg Tyr Arg Asp
            35   40   45

gtc agc cct ttt gac cac agt cgg att aaa ttg cat cag gaa gat aat   311
Val Ser Pro Phe Asp His Ser Arg Ile Lys Leu His Gln Glu Asp Asn
        50   55   60

gac tat atc aat gcc agc ttg ata aaa atg gag gaa gcc cag agg agc   359
Asp Tyr Ile Asn Ala Ser Leu Ile Lys Met Glu Glu Ala Gln Arg Ser
65   70   75   80

tat atc ctc acc cag ggc cct tta cca aac acg tgc ggg cac ttc tgg   407
Tyr Ile Leu Thr Gln Gly Pro Leu Pro Asn Thr Cys Gly His Phe Trp
                85   90   95

gag atg gtg tgg gag cag aag agc agg ggc gtg gtc atg ctc aac cgc   455
Glu Met Val Trp Glu Gln Lys Ser Arg Gly Val Val Met Leu Asn Arg
            100   105   110

atc atg gag aaa ggc tcg tta aaa tgt gcc cag tat tgg cca cag aaa   503
Ile Met Glu Lys Gly Ser Leu Lys Cys Ala Gln Tyr Trp Pro Gln Lys
            115   120   125

gaa gaa aaa gag atg gtc ttc gat gac acc aat ttg aag ctg aca ctg   551
Glu Glu Lys Glu Met Val Phe Asp Asp Thr Asn Leu Lys Leu Thr Leu
        130   135   140

```
atc tct gaa gat gtc aag tca tat tac aca gta cgg cag ttg gag ttg      599
Ile Ser Glu Asp Val Lys Ser Tyr Tyr Thr Val Arg Gln Leu Glu Leu
145             150                 155                 160

gag aac ctg gct acc cag gag gct cga gag atc ctg cat ttc cac tac      647
Glu Asn Leu Ala Thr Gln Glu Ala Arg Glu Ile Leu His Phe His Tyr
                165                 170                 175

acc acc tgg cct gac ttt gga gtc cct gag tca cct gcc tct ttc ctc      695
Thr Thr Trp Pro Asp Phe Gly Val Pro Glu Ser Pro Ala Ser Phe Leu
            180                 185                 190

aat ttc cta ttc aaa gtc cga gag tca ggc tca ctc agc cca gag cac      743
Asn Phe Leu Phe Lys Val Arg Glu Ser Gly Ser Leu Ser Pro Glu His
            195                 200                 205

ggc ccc att gtg gtc cac tgc agt gct ggc att ggc agg tca ggg acc      791
Gly Pro Ile Val Val His Cys Ser Ala Gly Ile Gly Arg Ser Gly Thr
    210                 215                 220

ttc tgc ctg gct gac acc tgc ctc tta ctg atg gac aag agg aaa gac      839
Phe Cys Leu Ala Asp Thr Cys Leu Leu Leu Met Asp Lys Arg Lys Asp
225             230                 235                 240

ccg tcc tct gtg gac atc aag aaa gtg ctg ttg gag atg cgc agg ttc      887
Pro Ser Ser Val Asp Ile Lys Lys Val Leu Leu Glu Met Arg Arg Phe
            245                 250                 255

cgc atg ggg ctc atc cag acg gcc gac caa ctg cgc ttc tcc tac ctg      935
Arg Met Gly Leu Ile Gln Thr Ala Asp Gln Leu Arg Phe Ser Tyr Leu
            260                 265                 270

gct gtg atc gag ggt gca aag ttc atc atg ggc gac tcg tca gtg cag      983
Ala Val Ile Glu Gly Ala Lys Phe Ile Met Gly Asp Ser Ser Val Gln
            275                 280                 285

gat cag tgg aag gag ctt tcc cat gaa gac ctg gag cct ccc cct gag     1031
Asp Gln Trp Lys Glu Leu Ser His Glu Asp Leu Glu Pro Pro Pro Glu
    290                 295                 300

cac gtg ccc cca cct ccc cgg cca ccc aaa cgc aca ttg gag cct cac     1079
His Val Pro Pro Pro Pro Arg Pro Pro Lys Arg Thr Leu Glu Pro His
305                 310                 315                 320

aat ggc aag tgc aag gag ctc ttc tcc aac cac cag tgg gtg agc gag     1127
Asn Gly Lys Cys Lys Glu Leu Phe Ser Asn His Gln Trp Val Ser Glu
            325                 330                 335

gag agc tgt gag gat gag gac atc ctg gcc aga gag gaa agc aga gcc     1175
Glu Ser Cys Glu Asp Glu Asp Ile Leu Ala Arg Glu Glu Ser Arg Ala
            340                 345                 350

ccc tca att gct gtg cac agc atg agc agt atg agt caa gac act gaa     1223
Pro Ser Ile Ala Val His Ser Met Ser Ser Met Ser Gln Asp Thr Glu
            355                 360                 365

gtt agg aaa cgg atg gtg ggt gga ggt ctt caa agt gct cag gca tct     1271
Val Arg Lys Arg Met Val Gly Gly Gly Leu Gln Ser Ala Gln Ala Ser
370                 375                 380
```

```
gtc ccc act gag gaa gag ctg tcc cca acc gag gag gaa caa aag gca     1319
Val Pro Thr Glu Glu Glu Leu Ser Pro Thr Glu Glu Glu Gln Lys Ala
385              390              395              400

cac agg cca gtt cac tgg aag ccc ttc ctg gtc aac gtg tgc atg gcc     1367
His Arg Pro Val His Trp Lys Pro Phe Leu Val Asn Val Cys Met Ala
             405              410              415

acg gcc ctg gcg act ggc gcg tac ctc tgt tac cgg gta tgt ttt cac     1415
Thr Ala Leu Ala Thr Gly Ala Tyr Leu Cys Tyr Arg Val Cys Phe His
             420              425              430

tga cagactgctg tgaggcatga gcgtggtggg cgctgccact gcccaggtta           1468
```

```
ggatttggtc tgcggcgtct aacctggtgt agaagaaaca acagcttaca agcctgtggt     1528

ggaactggaa gggccagccc caggaggggc atctgtgcac tgggctttga aggagcccct     1588

ggtcccaaga acagagtcta atctcagggc cttaacctgt tcaggagaag tagaggaaat     1648

gccaaatact cttcttgctc tcacctcact cctccccttt ctctggttcg tttgttttttg    1708

gaaaaaaaaa aaaaagaatt acaacacatt gttgttttta acatttataa aggcaggttt     1768

ttgttatttt tagagaaaac aaaagatgct aggcactggt gagattctct tgtgcccttt     1828

ggcatgtgat cagattcacg atttacgttt atttccgggg gagggtccca cctgtcagga     1888

ctgtaaagtt cctgctggct tggtcagccc ccccacccc ccaccccgag cttgcaggtg      1948

ccctgctgtg aggagagcag cagcagaggc tgcccctgga cagaagccca gctctgcttc     2008

cctcaggtgt ccctgcgttt ccatcctcct tctttgtgac cgccatcttg cagatgaccc     2068

agtcctcagc accccacccc tgcagatggg tttctccgag ggcctgcctc agggtcatca     2128

gaggttggct gccagcttag agctggggct tccatttgat tggaaagtca ttactattct     2188

atgtagaagc cactccactg aggtgtaaag caagactcat aaaggaggag ccttggtgtc     2248

atggaagtca ctccgcgcgc aggacctgta acaacctctg aaacactcag tcctgctgca     2308

gtgacgtcct tgaaggcatc agacagatga tttgcagact gccaagactt gtcctgagcc     2368

gtgatttttta gagtctggac tcatgaaaca ccgccgagcg cttactgtgc agcctctgat     2428

gctggttggc tgaggctgcg gggaggtgga cactgtgggt gcatccagtg cagttgcttt     2488

tgtgcagttg ggtccagcag cacagcccgc actccagcct cagctgcagg ccacagtggc     2548

catggaggcc gccagagcga gctggggtgg atgcttgttc acttggagca gccttcccag     2608

gacgtgcagc tcccttcctg ctttgtcctt ctgcttcctt ccctggagta gcaagcccac     2668

gagcaatcgt gagggggtgtg agggagctgc agaggcatca gagtggcctg cagcggcgtg     2728

aggccccttc ccctccgaca cccccctcca gaggagccgc tccactgtta tttattcact     2788
```

```
ttgcccacag acacccctga gtgagcacac cctgaaactg accgtgtaag gtgtcagcct    2848

gcacccagga ccgtcaggtg cagcaccggg tcagtcctag ggttgaggta ggactgacac    2908

agccactgtg tggctggtgc tggggcaggg gcaggagctg agggtcttag aagcaatctt    2968

caggaacaga caacagtggt gacatgtaaa gtccctgtgg ctactgatga catgtgtagg    3028

atgaaggctg gcctttctcc catgactttc tagatcccgt tccccgtctg ctttccctgt    3088

gagttagaaa acacacaggc tcctgtcctg gtggtgccgt gtgcttgaca tgggaaactt    3148

agatgcctgc tcactggcgg gcacctcggc atcgccacca ctcagagtga gagcagtgct    3208

gtccagtgcc gaggccgcct gactcccggc aggactcttc aggctctggc ctgccccagc    3268

acacccagct ggatctcaga cattccacac ccacacctca ttccctggac acttgggcaa    3328

gcaggcccgc ccttccacct ctggggtcag cccctccatt ccgagttcac actgctctgg    3388

agcaggccag gaccggaagc aaggcagctg gtgaggagca ccctcctggg aacagtgtag    3448

gtgacagtcc tgagagtcag cttgctagcg ctgctggcac cagtcacctt gctcagaagt    3508

gtgtggctct tgaggctgaa gagactgatg atggtgctca tgactcttct gtgaggggaa    3568

cttgaccttc acattgggtg gctttttta aaataagcga aggcagctgg aactccagtc    3628

tgcctcttgc cagcacttca cattttgcct ttcacccaga gaagccagca cagagccact    3688

ggggaaggcg atggccttgc ctgcacaggc tgaggagatg gctcagccgg cgtccaggct    3748

gtgtctggag cagggggtgc acagcagcct cacaggtggg ggcctcagag caggcgctgc    3808

cctgtcccct gccccgctgg aggcagcaaa gctgctgcat gccttaagtc aatacttact    3868

cagcagggcg ctctcgttct ctctctctct ctctctctct ctctctctct ctctctctct    3928

ctctctaaat ggccatagaa taaaccattt tacaaaaata aaagccaaca acaaagtgct    3988

ctggaatagc acctttgcag gagcgggggg tgtctcaggg tcttctgtga cctcaccgaa    4048

ctgtccgact gcaccgtttc caacttgtgt ctcactaatg ggtctgcatt agttgcaaca    4108

                                                                     4127
ataaatgttt ttaaagaac
```

```
<210> 11
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 11
cgagggtgca aagttcatca t                                                  21
```

```
<210> 12
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 12
ccaggtcttc atgggaaagc t                                    21


<210> 13
<211> 26
<212> DNA
<213> Artificial  Sequence

<220>
<223> PCR Probe

<400> 13
cgactcgtca gtgcaggatc agtgga                               26


<210> 14
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 14
tgttctagag acagccgcat ctt                                  23


<210> 15
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 15
caccgacctt caccatcttg t                                    21


<210> 16
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Probe

<400> 16
ttgtgcagtg ccagcctcgt ctca                                 24
```

```
<210> 17
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 17
cttagccccg aggcccgccc                                                         20


<210> 18
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 18
ctcggcccac tgcgccgtct                                                         20


<210> 19
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 19
catgacgggc cagggcggct                                                         20


<210> 20
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 20
cccggacttg tcgatctgct                                                         20


<210> 21
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 21
ctggcttcat gtcggatatc                                                         20
```

```
<210> 22
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 22
ttggccactc tacatgggaa                                                      20


<210> 23
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 23
ggactgacgt ctctgtacct                                                      20


<210> 24
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 24
gatgtagttt aatccgacta                                                      20


<210> 25
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 25
ctagcgttga tatagtcatt                                                      20


<210> 26
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 26
gggtaagaat gtaactcctt                                                      20
```

```
<210> 27
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 27
tgaccgcatg tgttaggcaa                                                    20


<210> 28
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 28
ttttctgctc ccacaccatc                                                   20


<210> 29
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 29
ctctgttgag catgacgaca                                                   20


<210> 30
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 30
gcgcatttta acgaaccttt                                                   20


<210> 31
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 31
aaatttgtgt cttcaaagat                                                   20
```

```
<210> 32
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 32
tgatatcttc agagatcaat                                                        20


<210> 33
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 33
tctagctgtc gcactgtata                                                        20


<210> 34
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 34
agtttcttgg gttgtaaggt                                                        20


<210> 35
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 35
gtggtatagt ggaaatgtaa                                                        20


<210> 36
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 36
tgattcaggg actccaaagt                                                        20
```

14

```
<210> 37
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 37
ttgaaaagaa agttcaagaa                                          20


<210> 38
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 38
gggctgagtg accctgactc                                          20


<210> 39
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 39
gcagtgcacc acaacgggcc                                          20


<210> 40
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 40
aggttccaga cctgccgatg                                          20


<210> 41
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 41
agcaggaggc aggtatcagc                                          20
```

```
<210> 42
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 42
gaagaagggt ctttcctctt                                          20


<210> 43
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 43
tctaacagca ctttcttgat                                          20


<210> 44
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 44
atcaacccca tccgaaactt                                          20


<210> 45
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 45
gagaagcgca gctggtcggc                                          20


<210> 46
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 46
tttggcacct tcgatcacag                                          20
```

```
<210> 47
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 47
agctccttcc actgatcctg                                                    20


<210> 48
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 48
tccaggattc gtttgggtgg                                                    20


<210> 49
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 49
gaactccctg catttcccat                                                    20


<210> 50
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 50
ttccttcacc cactggtgat                                                    20


<210> 51
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 51
gtagggtgcg gcatttaagg                                                    20
```

```
<210> 52
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 52
cagtgtcttg actcatgctt                                          20


<210> 53
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 53
gcctgggcac ctcgaagact                                          20


<210> 54
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 54
ctcgtccttc tcgggcagtg                                          20


<210> 55
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 55
gggcttccag taactcagtg                                          20


<210> 56
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 56
ccgtagccac gcacatgttg                                          20
```

```
<210> 57
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 57
tagcagaggt aagcgccggc                                                    20


<210> 58
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 58
ctatgtgttg ctgttgaaca                                                    20


<210> 59
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 59
ggaggtggag tggaggaggg                                                    20


<210> 60
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 60
ggctctgcgg gcagaggcgg                                                    20


<210> 61
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 61
ccgcggcatg cctgctagtc                                                    20
```

```
<210> 62
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 62
tctctacgcg gtccggcggc                                              20


<210> 63
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 63
aagatgggtt ttagtgcaga                                             20


<210> 64
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 64
gtactctctt tcactctcct                                             20


<210> 65
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 65
ggcccctccc ctctgcgccg                                             20


<210> 66
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 66
ctccaggagg gagccctggg                                             20
```

<210> 67
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 67
gggctgttgg cgtgcgccgc   20

<210> 68
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 68
tttaaataaa tatggagtgg   20

<210> 69
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 69
gttcaagaaa atgctagtgc   20

<210> 70
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 70
ttgataaagc ccttgatgca   20

<210> 71
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 71
atggcaaagc cttccattcc   20

```
<210> 72
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 72
gtcctccttc ccagtactgg                                    20


<210> 73
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 73
ttacccacaa tatcactaaa                                    20


<210> 74
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 74
attatatatt atagcattgt                                    20


<210> 75
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 75
tcacatcatg tttcttatta                                    20


<210> 76
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 76
ataacaggga ggagaataag                                    20
```

```
<210> 77
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 77
ttacatgcat tctaatacac                                              20


<210> 78
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 78
gatcaaagtt tctcatttca                                             20


<210> 79
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 79
ggtcatgcac aggcaggttg                                             20


<210> 80
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 80
caacaggctt aggaaccaca                                             20


<210> 81
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 81
aactgcaccc tattgctgag                                             20
```

```
<210> 82
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 82
gtcatgccag gaattagcaa                                                          20


<210> 83
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 83
acaggctggg cctcaccagg                                                          20


<210> 84
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 84
tgagttacag caagaccctg                                                          20


<210> 85
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 85
gaatatggct tcccataccc                                                          20


<210> 86
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 86
ccctaaatca tgtccagagc                                                          20
```

```
<210> 87
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 87
gacttggaat ggcggaggct                                                          20


<210> 88
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 88
caaatcacgg tctgctcaag                                                          20


<210> 89
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 89
gaagtgtggt ttccagcagg                                                          20


<210> 90
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 90
cctaaaggac cgtcacccag                                                          20


<210> 91
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 91
gtgaaccggg acagagacgg                                                          20
```

```
<210> 92
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 92
gccccacagg gtttgagggt                                                    20


<210> 93
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 93
cctttgcagg aagagtcgtg                                                    20


<210> 94
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 94
aaagccactt aatgtggagg                                                    20


<210> 95
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 95
gtgaaaatgc tggcaagaga                                                    20


<210> 96
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 96
tcagaatgct tacagcctgg                                                    20
```

```
<210> 97
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 97
caacctcccc agcagcggct                                        20


<210> 98
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 98
tcgaggcccg tcgcccgcca                                        20


<210> 99
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 99
cctcggccgt ccgccgcgct                                        20


<210> 100
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 100
tcgatctgct cgaattcctt                                        20


<210> 101
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 101
cctggtaaat agccgcccag                                        20
```

```
<210> 102
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 102
tgtcgaatat cctggtaaat                                              20


<210> 103
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 103
actggcttca tgtcgaatat                                              20


<210> 104
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 104
aagtcactgg cttcatgtcg                                              20


<210> 105
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 105
gaagtcactg gcttcatgtc                                              20


<210> 106
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 106
ggaagtcact ggcttcatgt                                              20
```

```
<210> 107
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 107
gggaagtcac tggcttcatg                                        20


<210> 108
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 108
tgggaagtca ctggcttcat                                        20


<210> 109
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 109
atgggaagtc actggcttca                                        20


<210> 110
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 110
catgggaagt cactggcttc                                        20


<210> 111
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 111
tttttgttct taggaagttt                                        20
```

```
<210> 112
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 112
cggttttttgt tcttaggaag                                                          20


<210> 113
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 113
tccgactgtg gtcaaaaggg                                                           20


<210> 114
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 114
ttaatccgac tgtggtcaaa                                                           20


<210> 115
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 115
atagtcatta tcttcctgat                                                           20


<210> 116
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 116
ttgatatagt cattatcttc                                                           20
```

```
<210> 117
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 117
gcttcctcca tttttatcaa                                        20


<210> 118
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 118
ggccctgggt gaggatatag                                        20


<210> 119
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 119
cacaccatct cccagaagtg                                        20


<210> 120
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 120
tgctcccaca ccatctccca                                        20


<210> 121
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 121
ctgctcccac accatctccc                                        20
```

```
<210> 122
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 122
tctgctccca caccatctcc                                                              20


<210> 123
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 123
ttctgctccc acaccatctc                                                              20


<210> 124
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 124
cccctgctct tctgctccca                                                              20


<210> 125
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 125
atgcggttga gcatgaccac                                                              20


<210> 126
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 126
tttaacgagc ctttctccat                                                              20
```

```
<210> 127
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 127
ttttcttctt tctgtggcca                                              20


<210> 128
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 128
gaccatctct ttttcttctt                                              20


<210> 129
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 129
tcagagatca gtgtcagctt                                              20


<210> 130
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 130
cttgacatct tcagagatca                                              20


<210> 131
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 131
taatatgact tgacatcttc                                              20
```

<210> 132
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 132
aactccaact gccgtactgt   20

<210> 133
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 133
tctctcgagc ctcctgggta   20

<210> 134
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 134
ccaaagtcag gccaggtggt   20

<210> 135
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 135
gggactccaa agtcaggcca   20

<210> 136
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 136
agggactcca aagtcaggcc   20

34

```
<210> 137
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 137
cagggactcc aaagtcaggc                                              20


<210> 138
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 138
tcagggactc caaagtcagg                                              20


<210> 139
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 139
ggtgactcag ggactccaaa                                              20


<210> 140
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 140
cctgactctc ggactttgaa                                              20


<210> 141
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 141
gctgagtgag cctgactctc                                              20
```

<210> 142
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 142
ccgtgctctg ggctgagtga   20

<210> 143
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 143
aaggtccctg acctgccaat   20

<210> 144
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 144
tctttcctct tgtccatcag   20

<210> 145
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 145
gtctttcctc ttgtccatca   20

<210> 146
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 146
ggtctttcct cttgtccatc   20

```
<210> 147
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 147
gggtctttcc tcttgtccat                                              20


<210> 148
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 148
aacagcactt tcttgatgtc                                              20


<210> 149
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 149
ggaacctgcg catctccaac                                              20


<210> 150
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 150
tggtcggccg tctggatgag                                              20


<210> 151
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 151
gagaagcgca gttggtcggc                                              20
```

```
<210> 152
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 152
aggtaggaga agcgcagttg                                    20


<210> 153
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 153
gccaggtagg agaagcgcag                                    20


<210> 154
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 154
agccaggtag gagaagcgca                                    20


<210> 155
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 155
cagccaggta ggagaagcgc                                    20


<210> 156
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 156
acagccaggt aggagaagcg                                    20
```

```
<210> 157
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 157
cacagccagg taggagaagc                                                    20


<210> 158
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 158
tcacagccag gtaggagaag                                                    20


<210> 159
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 159
atcacagcca ggtaggagaa                                                    20


<210> 160
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 160
gatcacagcc aggtaggaga                                                    20


<210> 161
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 161
cgatcacagc caggtaggag                                                    20
```

<210> 162
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 162
tcgatcacag ccaggtagga   20

<210> 163
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 163
caccctcgat cacagccagg   20

<210> 164
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 164
tccttccact gatcctgcac   20

<210> 165
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 165
ctccttccac tgatcctgca   20

<210> 166
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 166
gctccttcca ctgatcctgc   20

```
<210> 167
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 167
agctccttcc actgatcctg                                          20


<210> 168
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 168
aagctccttc cactgatcct                                          20


<210> 169
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 169
aaagctcctt ccactgatcc                                          20


<210> 170
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 170
gaaagctcct tccactgatc                                          20


<210> 171
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 171
ggaaagctcc ttccactgat                                          20
```

```
<210> 172
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 172
gggaaagctc cttccactga                                                                      20


<210> 173
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 173
tgggaaagct ccttccactg                                                                      20


<210> 174
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 174
tggccgggga ggtgggggca                                                                      20


<210> 175
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 175
tgggtggccg gggaggtggg                                                                      20


<210> 176
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 176
tgcgtttggg tggccgggga                                                                      20
```

```
<210> 177
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 177
tgcacttgcc attgtgaggc                                               20


<210> 178
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 178
acttcagtgt cttgactcat                                               20


<210> 179
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 179
aacttcagtg tcttgactca                                               20


<210> 180
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 180
taacttcagt gtcttgactc                                               20


<210> 181
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 181
ctaacttcag tgtcttgact                                               20
```

<210> 182
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 182
gacagatgcc tgagcacttt   20

<210> 183
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 183
gaccaggaag ggcttccagt   20

<210> 184
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 184
tgaccaggaa gggcttccag   20

<210> 185
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 185
ttgaccagga agggcttcca   20

<210> 186
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 186
gttgaccagg aagggcttcc   20

<210> 187
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 187
gcacacgttg accaggaagg   20

<210> 188
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 188
gaggtacgcg ccagtcgcca   20

<210> 189
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 189
tacccggtaa cagaggtacg   20

<210> 190
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 190
agtgaaaaca tacccggtaa   20

<210> 191
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 191
caaatcctaa cctgggcagt   20

```
<210> 192
<211> 20   .          .
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 192
ttccagttcc accacaggct                                                      20


<210> 193
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 193
ccagtgcaca gatgcccctc                                                      20


<210> 194
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 194
acaggttaag gccctgagat                                                      20


<210> 195
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 195
gcctagcatc ttttgttttc                                                      20


<210> 196
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 196
aagccagcag gaactttaca                                                      20
```

```
<210> 197
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 197
gggacacctg agggaagcag                                                 20


<210> 198
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 198
ggtcatctgc aagatggcgg                                                 20


<210> 199
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 199
gccaacctct gatgaccctg                                                 20


<210> 200
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 200
tggaagcccc agctctaagc                                                 20


<210> 201
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 201
tagtaatgac tttccaatca                                                 20
```

```
<210> 202
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 202
tgagtcttgc tttcacacctc                                            20


<210> 203
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 203
cctgcgcgcg gagtgacttc                                             20


<210> 204
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 204
aggacgtcac tgcagcagga                                             20


<210> 205
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 205
tcaggacaag tcttggcagt                                             20


<210> 206
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 206
gaggctgcac agtaagcgct                                             20
```

```
<210> 207
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 207
tcagccaacc agcatcagag                                                    20


<210> 208
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 208
acccacagtg tccacctccc                                                    20


<210> 209
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 209
agtgcgggct gtgctgctgg                                                    20


<210> 210
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 210
cagctcgctc tggcggcctc                                                    20


<210> 211
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 211
aggaagggag ctgcacgtcc                                                    20
```

```
<210> 212
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 212
ccctcacgat tgctcgtggg                                                 20


<210> 213
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 213
cagtggagcg gctcctctgg                                                 20


<210> 214
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 214
caggctgaca ccttacacgg                                                 20


<210> 215
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 215
gtcctacctc aaccctagga                                                 20


<210> 216
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 216
ctgccccagc accagccaca                                                 20
```

```
<210> 217
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 217
attgcttcta agaccctcag                                    20


<210> 218
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 218
ttacatgtca ccactgttgt                                    20


<210> 219
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 219
tacacatgtc atcagtagcc                                    20


<210> 220
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 220
ttttctaact cacagggaaa                                    20


<210> 221
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 221
gtgcccgcca gtgagcaggc                                    20
```

```
<210> 222
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 222
cggcctcggc actggacagc                                                    20


<210> 223
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 223
gtggaatgtc tgagatccag                                                    20


<210> 224
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 224
agggcgggcc tgcttgccca                                                    20


<210> 225
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 225
cggtcctggc ctgctccaga                                                    20


<210> 226
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 226
tacactgttc ccaggagggt                                                    20
```

<210> 227
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 227
tggtgccagc agcgctagca   20

<210> 228
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 228
cagtctcttc agcctcaaga   20

<210> 229
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 229
aagagtcatg agcaccatca   20

<210> 230
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 230
tgaaggtcaa gttcccctca   20

<210> 231
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 231
ctggcaagag gcagactgga   20

<210> 232
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 232
ggctctgtgc tggcttctct   20

<210> 233
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 233
gccatctcct cagcctgtgc   20

<210> 234
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 234
agcgcctgct ctgaggcccc   20

<210> 235
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 235
tgctgagtaa gtattgactt   20

<210> 236
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 236
ctatggccat ttagagagag   20

```
<210> 237
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 237
tggtttattc tatggccatt                                                       20


<210> 238
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 238
cgctcctgca aaggtgctat                                                       20


<210> 239
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 239
gttggaaacg gtgcagtcgg                                                       20


<210> 240
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 240
atttattgtt gcaactaatg                                                       20


<210> 241
<211> 2346
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (710)...(2008)

<400> 241
gaattcggga tccttttgca cattcctagt tagcagtgca tactcatcag actggagatg 60
```

tttaatgaca tcagggaacc aaacggacaa cccatagtac ccgaagacag ggtgaaccag 120

acaatcgtaa gcttgatggt gttttccctg actgggtagt tgaagcatct catgaatgtc 180

agccaaattc cgtacagttc ggtgcggatc cgaacgaaac acctcctgta ccaggttccc 240

gtgtcgctct caatttcaat cagctcatct atttgtttgg gagtcttgat tttatttacc 300

gtgaagacct tctctggctg gccccgggct ctcatgttgg tgtcatgaat taacttcaga 360

atcatccagg cttcatcatg ttttcccacc tccagcaaga accgagggct ttctggcatg 420

aaggtgagag ccaccacaga ggagacgcat gggagcgcac agacgatgac gaagacgcgc 480

cacgtgtgga actggtaggc tgaacccatg ctgaagctcc accgtagtg gggaatgatg 540

gcccaggcat ggcggaggct agatgccgcc aatcatccag aacatgcaga agccgctgct 600

ggggagcttg gggctgcggt ggtggcgggt gacgggcttc gggacgcgga gcgacgcggc 660

ctagcgcggc ggacggccgt gggaactcgg gcagccgacc cgtcccgcc atg gag atg 718
                                                          Met Glu Met

                                                               1

gag aag gag ttc gag gag atc gac aag gct ggg aac tgg gcg gct att   766
Glu Lys Glu Phe Glu Glu Ile Asp Lys Ala Gly Asn Trp Ala Ala Ile
        5   10   15

tac cag gac att cga cat gaa gcc agc gac ttc cca tgc aaa gtc gcg   814
Tyr Gln Asp Ile Arg His Glu Ala Ser Asp Phe Pro Cys Lys Val Ala
    20   25   30   35

aag ctt cct aag aac aaa aac cgg aac agg tac cga gat gtc agc cct   862
Lys Leu Pro Lys Asn Lys Asn Arg Asn Arg Tyr Arg Asp Val Ser Pro
                    40   45   50

ttt gac cac agt cgg att aaa ttg cac cag gaa gat aat gac tat atc   910
Phe Asp His Ser Arg Ile Lys Leu His Gln Glu Asp Asn Asp Tyr Ile
                55   60   65

aat gcc agc ttg ata aaa atg gaa gaa gcc cag agg agc tat att ctc   958
Asn Ala Ser Leu Ile Lys Met Glu Glu Ala Gln Arg Ser Tyr Ile Leu
            70   75   80

acc cag ggc cct tta cca aac aca tgt ggg cac ttc tgg gag atg gtg   1006

56

Thr Gln Gly Pro Leu Pro Asn Thr Cys Gly His Phe Trp Glu Met Val
    85   90   95

tgg gag cag aag agc agg ggc gtg gtc atg ctc aac cgc atc atg gag   1054
Trp Glu Gln Lys Ser Arg Gly Val Val Met Leu Asn Arg Ile Met Glu
100   105   110   115

aaa ggc tcg tta aaa tgt gcc cag tat tgg cca cag caa gaa gaa aag   1102
Lys Gly Ser Leu Lys Cys Ala Gln Tyr Trp Pro Gln Gln Glu Glu Lys
                120   125   130

gag atg gtc ttt gat gac aca ggt ttg aag ttg aca cta atc tct gaa   1150
Glu Met Val Phe Asp Asp Thr Gly Leu Lys Leu Thr Leu Ile Ser Glu
                135   140   145

gat gtc aag tca tat tac aca gta cga cag ttg gag ttg gaa aac ctg   1198
Asp Val Lys Ser Tyr Tyr Thr Val Arg Gln Leu Glu Leu Glu Asn Leu
            150   155   160

act acc aag gag act cga gag atc ctg cat ttc cac tac acc aca tgg   1246
Thr Thr Lys Glu Thr Arg Glu Ile Leu His Phe His Tyr Thr Thr Trp
    165   170   175

cct gac ttt gga gtc ccc gag tca ccg gct tct ttc ctc aat ttc ctt   1294
Pro Asp Phe Gly Val Pro Glu Ser Pro Ala Ser Phe Leu Asn Phe Leu
180   185   190   195

ttc aaa gtc cga gag tca ggc tca ctc agc ctg gag cat ggc ccc att   1342
Phe Lys Val Arg Glu Ser Gly Ser Leu Ser Leu Glu His Gly Pro Ile
                200   205   210

gtg gtc cac tgc agc gcc ggc atc ggg agg tca ggg acc ttc tgt ctg   1390
Val Val His Cys Ser Ala Gly Ile Gly Arg Ser Gly Thr Phe Cys Leu
                215   220   225

gct gac acc tgc ctc tta ctg atg gac aag agg aaa gac cca tct tcc   1438
Ala Asp Thr Cys Leu Leu Leu Met Asp Lys Arg Lys Asp Pro Ser Ser
            230   235   240

```
gtg gac atc aag aaa gta ctg ctg gag atg cgc agg ttc cgc atg ggg      1486
Val Asp Ile Lys Lys Val Leu Leu Glu Met Arg Arg Phe Arg Met Gly
    245             250             255

ctc atc cag act gcc gac cag ctg cgc ttc tcc tac ctg gct gtc atc      1534
Leu Ile Gln Thr Ala Asp Gln Leu Arg Phe Ser Tyr Leu Ala Val Ile
260             265             270             275

gag ggc gcc aag ttc atc atg ggc gac tcg tca gtg cag gat cag tgg      1582
Glu Gly Ala Lys Phe Ile Met Gly Asp Ser Ser Val Gln Asp Gln Trp
                280             285             290

aag gag ctc tcc cgg gag gat cta gac ctt cca ccc gag cac gtg ccc      1630
Lys Glu Leu Ser Arg Glu Asp Leu Asp Leu Pro Pro Glu His Val Pro
            295             300             305

cca cct ccc cgg cca ccc aaa cgc aca ctg gag cct cac aac ggg aag      1678
Pro Pro Pro Arg Pro Pro Lys Arg Thr Leu Glu Pro His Asn Gly Lys
            310             315             320

tgc aag gag ctc ttc tcc agc cac cag tgg gtg agc gag gag acc tgt      1726
Cys Lys Glu Leu Phe Ser Ser His Gln Trp Val Ser Glu Glu Thr Cys
            325             330             335

ggg gat gaa gac agc ctg gcc aga gag gaa ggc aga gcc cag tca agt      1774
Gly Asp Glu Asp Ser Leu Ala Arg Glu Glu Gly Arg Ala Gln Ser Ser
340             345             350             355

gcc atg cac agc gtg agc agc atg agt cca gac act gaa gtt agg aga      1822
Ala Met His Ser Val Ser Ser Met Ser Pro Asp Thr Glu Val Arg Arg
            360             365             370

cgg atg gtg ggt gga ggt ctt caa agt gct cag gcg tct gtc ccc acc      1870
Arg Met Val Gly Gly Gly Leu Gln Ser Ala Gln Ala Ser Val Pro Thr
            375             380             385

gag gaa gag ctg tcc tcc act gag gag gaa cac aag gca cat tgg cca      1918
Glu Glu Glu Leu Ser Ser Thr Glu Glu Glu His Lys Ala His Trp Pro
            390             395             400
```

agt cac tgg aag ccc ttc ctg gtc aat gtg tgc atg gcc acg ctc ctg   1966
Ser His Trp Lys Pro Phe Leu Val Asn Val Cys Met Ala Thr Leu Leu
     405   410   415

gcc acc ggc gcg tac ttg tgc tac cgg gtg tgt ttt cac tga   2008
Ala Thr Gly Ala Tyr Leu Cys Tyr Arg Val Cys Phe His *
420   425   430

cagactggga ggcactgcca ctgcccagct taggatgcgg tctgcggcgt ctgacctggt 2068

gtagagggaa caacaactcg caagcctgct ctggaactgg aagggcctgc cccaggaggg 2128

tattagtgca ctgggctttg aaggagcccc tggtcccacg aacagagtct aatctcaggg 2188

ccttaacctg ttcaggagaa gtagaggaaa tgccaaatac tcttcttgct ctcacctcac 2248

tcctcccctt tctctgattc atttgttttt ggaaaaaaaa aaaaaaagaa ttacaacaca 2308

ttgttgtttt taacatttat aaaggcaggc ccgaattc   2346

<210> 242
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<221> unsure
<222> (1)..(20)
<223> Antisense Oligonucleotide

<400> 242
nnnnnnnnnn nnnnnnnnnn   20